AD______________

Award Number DAMD17-96-1-6223

TITLE: Development of PEA3 as a Potential Gene Therapy Agent for Breast Cancer

PRINCIPAL INVESTIGATOR: Mr. Zhenming Yu

CONTRACTING ORGANIZATION: University of Texas
M. D. Anderson Cancer Center
Houston, Texas 77030

REPORT DATE: October 1999

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1999	Final (1 Sep 96 - 31Aug 99)

4. TITLE AND SUBTITLE
Development of PEA3 as a Potential Gene Therapy Agent for Breast Cancer

5. FUNDING NUMBERS
DAMD17-96-1-6223

6. AUTHOR(S)
Mr. Zhenming Yu

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Texas M. D. Anderson Cancer Center
Houston, Texas 77030

E-mail: zyu@gsbs3.gs.uth.tmc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Deterick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

HER-2/neu overexpression plays an important role for cancer development. To suppress cell transformation mediated by HER-2/neu overexpression, we identified a DNA-binding protein PEA3 of the Ets gene family which specifically targeted a DNA sequence on the HER2/neu promoter and downregulated the promoter activity. Expression of PEA3 resulted in preferential inhibition of cell growth and tumor development of HER-2/neu-overexpressing cancer cells. A competition model to elucidate the molecular mechanism for the transcription inhibition of Her-2/neu by PEA3 was proposed. PEA3 directly binds to the HER-2/neu promoter and competition of PEA3 with other Ets transactivating factor(s) for a positive regulatory motif on the Her-2/neu promoter may acount for the downregulation of Her-2/neu transcription by PEA3.

14. SUBJECT TERMS
Breast Cancer, PEA3, Her-2/neu, Transcriptional Repression, Tumor Suppression.

15. NUMBER OF PAGES
73

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclasified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

_✓_ Where copyrighted material is quoted, permission has been obtained to use such material.

_✓_ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

_✓_ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

_✓_ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

_✓_ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

_✓_ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

_✓_ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Sheming Lu 9/20/99

PI - Signature Date

TABLE OF CONTENTS

INTRODUCTION

Breast cancer is one of the major causes of death for women in the United States (1,2). Amplification/overexpression of the Her-2/neu gene was found in approximately 30% of human breast cancer (3-8). Overexpression of the Her-2/neu oncogene correlates with poor survival for breast cancer patients and enhances metastatic potential and chemoresistance of human cancer cells (3, 8, 9, 10, 11). Downregulation of Her-2/neu suppresses the malignant phenotypes of cancer cells with Her-2/neu overexpression, indicating that Her-2/neu oncogene is an ideal target for development of the novel therapeutic agent (12-17). In this study, we found that PEA3, a member of the Ets transcriptional factor family, could directly bind to the consensus motif on the Her-2/neu promoter and prevent Her-2/neu gene overexpression by suppressing the promoter activity. Downregulation of Her-2/neu expression by PEA3 resulted in reversing the transforming phenotype mediated by Her-2/neu overexpression, inhibiting cell growth *in vitro*, and, in a preclinical gene therapy setting, blocking tumor formation from Her-2/neu-overexpressing cancer cells and prolonging survival rate of treated animals.

BODY

1. The tasks outlined in the approved Statement of Work:

Task 1. Examination of tumor suppression activity of PEA3 in breast cancer cell lines. Months 3-8.

Task 2. Examination of the anti-tumor effect of PEA3 by using liposome-mediated gene transfer. Months13-24.

Task 3. Construction of adenovirus vector carrying PEA3, and control without PEA3. Infection of human breast cancer cell lines and examination of *in vitro* and *in vivo* transformation phenotype of these infected cells, including cell growth rate, colony formation ability in soft agar and turnorigenicity of m.f.p. injection. Months 13-36.

Task 4. Determining of the therapeutic effect of Ad.PEA3 in HER-2/neu-overexpressing breast cancers. Months 12-24.

Task 5. Testing the synergistic effect of PEA3-mediated gene therapy and chemotherapy *in vitro* and *in vivo*. Months 24-48.

2. The research accomplishment to date with respect to the Statement of Work:

A. <u>We have completed Task1 and Task 2. The results are as the following:</u>

(1). PEA3 suppress the HER-2/neu transforming phenotype *in vitro*

To test whether expression of PEA3 can repress HER-2/neu expression and therefore reverse the transforming phenotype, a genomic activated rat neu oncogene (cNu104) that was known to transform mouse fibroblast cells and result in enhanced focus-forming activity (18-20) was

subject to a focus forming assay by cotransfecting with the PEA3 cDNA or the control vector into the mouse fibroblast NIH3T3 cells. The results indicated that PEA3 dramatically suppressed the focus formation caused by cNu104 (Fig. 1A and B).

To test if ectopic expression of PEA3 can suppress cell growth of cancer cells, breast and ovarian cancer cells with high or basal level HER-2/neu expression were transfected with a plasmid carrying a neomycin resistance gene and the PEA3 cDNA controlled by a CMV promoter. Subsequent neomycin selection resulted in numerous resistant colonies from the HER-2/neu low expressing cell lines while only few colonies of cell lines with HER-2/neu overexpression were retained through the selection (Fig. 1C and Table 1). The surviving colonies were isolated and tested for ectopic expression of PEA3 by RT-PCR. Virtually all of the clones derived from the HER-2/neu low-expression cell line (MDA-MB-435), while only two clones derived from the HER-2/neu 6 overexpressing cell line (MDA-NO-453), contained PEA3 mRNA expressed from the plasmid (Fig. 1D). One of the two clones from MDA-MB-453 (clone No. 2) did not express detectable PEA3 protein. Another clone (clone No. 8) expressing ectopic PEA3 protein grew extremely slow and eventually lost during subsequent cell culturing (data not shown). The facts that transfection of PEA3 gene into HER-2/neu-overexpressing cells reduces the number of neomycin-resistant colonies and that the survived clones either do not express PEA3 protein or are unable to grow in cell culture strongly suggest that PEA3 inhibits growth of HER-2/neu-overexpressing cancer cells.

(2). PEA3 inhibits tumor growth *in vivo*

To further investigate the tumor suppression potential of PEA3, we used an established animal model to test whether PEA3 could suppress tumor development in animals and prolong animal survival (17, 21). Ovarian cancer orthotopic tumor xenografts derived from a HER-2/neu-overexpressing cell line (SKOV3-ipl) or a cell line with only basal level of HER-2/neu expression (2774c-10) were developed intraperitoneally in nude mice. Tumor-bearing mice were treated with or without PEA3-expressing plasmid DNA once per week delivered by a cationic liposome (DC-Chol) by intraperitoneal injection. Fifty percent of mice with SKOV3-ipl tumors survived without detectable tumors for longer than one year after treated by PEA3 plus liposome, while all mice in the control groups died within half year (Fig. 2A). On the other hand, no response to PEA3-liposome treatment was observed for mice bearing 2774c-10-derived tumors

(Fig. 2B). The therapeutic effect of PEA3 combined with the cationic liposome was associated with down-regulation of the HER-2/neu p185 protein product in the treated tumors of SKOV3-ipl xenograft (Fig. 2A insert).

B. To achieve the goals in Task 3 and Task 4, we had tried to construct the adenovirus vector carrying PEA3. However, we could not get the infectious virus carrying PEA3 so far.

We suspect that this is due to the growth inhibition effect of PEA3 on the recipient cell. Indeed, to our experience, we had not been able to obtain the infectious virus carrying pro-apoptotic gene Bik/Nbk either.

We would like to focus on using PEA3-liposome complex instead of adenovirus construct for therapy purpose later on.

C. Experiments proposed in Task 5 has just been initiated and we do not have any conclusive data to date.

We will search for other funding to continue our study on this important issue.

3. Besides the tasks outlined in the Statement of Work, we further investigated the molecular mechanism for PEA3 to repress the Her-2/neu promoter activity. We also found that DNA binding domain of PEA3 along is sufficient to repress Her-2/neu promoter. The results are presented as the following:

A. PEA3 binds to the HER-2/neu promoter

A DNA motif with the sequence of 5'AGGAAG3' has been identified on the HER-2/neu promoter (22). To test whether PEA3 can recognize and bind to this putative PEA3 binding site, purified GST-PEA3 fusion protein was prepared and incubated with ^{32}p-labeled oligonucleotide probes containing either the wild-type PEA3 binding site sequence or the same sequence but with the core PEA3 binding motif mutated (5'AGCTCG3'). DNA-protein association was

investigated with an electrophoretic mobility shift assay (EMSA). Specific binding between the fusion protein and the wild-type probe was identified (Fig. 3). The binding was diminished in the presence of unlabeled wild-type oligonucleotide. There was no detectable association between the fusion protein and the mutant oligonucleotide. GST alone does not bind to the probe (data not shown).

B. <u>PEA3 represses the HER-2/neu promoter activity through a positive regulatory motif of the promoter</u>

To investigate the effect of PEA3 on the promoter activity of HER-2/neu, luciferase reporter gene driven by the HER-2/neu promoter (pNulit) was cotransfected with different amount of PEA3 cDNA into the SKOV-3 ovarian cancer cell line (Fig. 4A). The PEA3 cDNA repressed the promoter activity of HER-2/neu in a dose-dependent manner. Similar results were observed in experiments using a breast cancer cell line MDA-MB-453. In these experiments both the luciferase (Fig. 4A, 5A) and the chloramphenical acetyl transferase (HER2-CAT; Fig. 4B) reporter genes consistently demonstrated that PEA3 co-expression can repress the HER-2/neu promoter activity. These results indicate that PEA3 is a potent transrepressor for HER-2/neu gene expression. The intact PEA3 binding site on the HER-2/neu promoter is required for PEA3-mediated transcriptional repression because the HER-2/neu promoter with a mutated PEA3 binding sequence (5'AGGAAG3' to 5'AGCTCG3') was not subject to the negative regulation by PEA3 (Fig. 5A). The results suggest that PEA3-mediated HER2/neu down-regulation is through the PEA3 binding site on the HER-2/neu promoter. When the promoter activities were compared between the wild-type and mutant promoters in the absence of PEA3, the activity of the mutant promoter was significantly lower than that of the wild-type promoter (Fig. 5B), indicating that the PEA3 binding site on the HER-2/neu promoter actually acts as a positive regulatory element for elevated expression of HER-2/neu.

C. <u>PEA3 competes with other transactivating factor(s)</u>

The results shown above demonstrate that PEA3 down-regulates HER-2/neu expression and suppresses the transforming phenotype mediated by HER-2/neu overexpression. The fact that the PEA3 binding motif on the HER-2/neu promoter functions as a positive regulatory element for HER-2/neu gene transcription (Fig. 5B) implicates the presence of a yet unidentified

transactivating factor(s) which can recognize the same positive regulatory element and that PEA3 may compete with the transactivating factor(s) for the same DNA motif and result in repression of HER-2/neu transcription. To test this hypothesis, nuclear extract from the HER-2/neuoverexpressing cell line MDA-MB-453 was prepared and tested for binding activity to a oligonucleotide probe containing DNA sequence of the PEA3 binding site in the presence of increasing amount of GST-PEA3 fusion protein. The EMSA results revealed a specific nuclear binding activity to the PEA3 binding site (Fig. 6A). The binding of the unknown factor(s) is unique and can be distinguished from the PEA3-DNA complex. Furthermore, increasing doses of PEA3 fusion protein diminished the unique binding activity in MDA-MB-453 cells (Fig. 6B), indicating a competition relationship between PEA3 and the unidentified transcriptional factor(s). This competition model suggests that overexpression of the DNA-binding activity of PEA3 would be sufficient to suppress HER-2/neu gene expression by blocking the binding of other transactivators to the same DNA motif. To test this hypothesis, a PEA3 construct containing only the DNA binding domain was cotransfected with pNulit into MDA-MB-453 cells. Expression of the DNA binding domain of PEA3 (PEA3DBD) resulted in decrease of the luciferase reporter activity in a dose-dependent manner (Fig. 6Q. Thus, consistent with the hypothesis, either wild-type PEA3 or the DNA-binding domain of PEA3 can function as potent repressors of the HER-2/neu gene expression -

4. Discussion

(1). PEA3 as an Ets family protein

The PEA3 protein contains a domain of about 85 amino acids with extensive sequence similarity with the ETS domain, a conserved region shared by all members of the Ets family that characteristically bind to the cognate DNA binding site as a monomer through their ETS DNA-binding domain. The consensus DNA binding site of PEA3 seems to occur on the promoters of a cohort of genes and play a positive role for their expression. Examples include the stromelysine gene (23) and the tumor suppressor gene maspin (24, 25). For most of the cases, there are other Ets proteins rather than the PEA3 protein itself shown to target the consensus binding site and regulate the expression of the genes. The biological effects of the PEA3 protein to these genes remain to be determined. This study, to our knowledge, for the first

time shows the tumor suppression function of an Ets protein through suppressing the oncogenic activity of the HER-2/neu oncogene.

(2). The PEA3 protein down-regulates HER-2/neu promoter

We have identified a DNA motif on the HER-2/neu promoter that directly binds to the Ets-related transcription factor PEA3 and results in down-regulation of the HER-2/neu gene and consequently inhibition of cell transformation *in vitro* and tumor development *in vivo*. Our results indicated that PEA3 might act as a transcriptional repressor and likely compete with other transactivators through the same DNA motif. Since some other Ets family members which share the same binding site specificity as PEA3 may also bind to the same PEA3 binding site on the HER-2/neu promoter and transactivate the gene, it is conceivable that PEA3 may repress the HER-2/neu promoter by blocking the binding of other transactivating Ets proteins. There are precedents of other Ets family members, like ERF and Net, acting as repressors of gene expression (26, 27). On the other hand, promoters negatively regulated by Ets binding sites have also been reported (28, 29).

The effect of PEA3 on HER-2/neu promoter in this study is observed mainly in human breast and ovarian cancer cell lines, which are biologically relevant to the diseases. Other groups have previously reported transactivation of HER-2/neu promoter by PEA3 in COS-1 cells (30). Under our experimental condition, in COS- 1 cells we did notice a very weak PEA3-mediated induction of HER-2/neu promoter. The potential species- or cell type-specific transactivating effect of PEA3 is an interesting phenomenon. It might be due to the disparity of regulatory mechanisms between different cell types and/or species. In this regard, it is worth noting that COS-1 cells were derived from monkey kidney cells transformed by SV40 T antigen (31).

(3). The PEA3 gene as a potential gene therapy agent

Recently targeting the protein product of HER-2/neu p185 with a humanized anti-p185 monoclonal antibody (Herceptin) has shown encouraging therapeutic effects for patients with HER-2/neu-overexpressing breast cancer though with significant side effect of cardiotoxicity (32, 33). The results, however, indicate that HER-2/neu overexpression is an excellent target for development of therapeutic strategies of cancer. In addition to the immunotherapy strategy, a

gene-therapy setting using either the adenovirus type 5 E1A or the SV40 large T antigen gene to down-regulate HER-2/neu overexpression has been reported (12,16,17,21,30, 34). In the case of E1A, a phase I clinical trial has just been completed with observation of HER-2/neu down-regulation associated with EIA expression (35). However, both E1A and SV40 large T antigen are viral proteins and very likely suppress HER-2/neu in an indirect manner as none of these proteins have been shown to bind the HER-2/neu promoter directly. The fact that PEA3 directly binds to the HER-2/neu promoter likely provides stronger efficacy and higher specificity to inhibit HER-2/neu promoter activity. We have presented the data to demonstrate that the DNA binding domain of PEA3 is sufficient to down-regulate HER-2/neu overexpression. These features make PEA3 an attractive candidate for further molecular manipulation to develop next generation therapeutic molecules with higher binding affinity and enhanced specificity. In addition, the strategy described in this study provides a general approach to identify potential transformation suppressors through searching for DNA-binding protein recognizing specific DNA sequence on the promoter of the targeted oncogenes or other disease-causing pathogenic genes.

5. Future work to better address this topic

(1). Test whether p300/CBP plays a similar role in PEA3-mediated inhibition of Her-2/neu transcription as in E1A-mediated inhibition of Her-2/neu transcription.

It was reported that p300/CBP interacts with Ets-1 and Ets-2 in the transcription activation of the human stromelysin promoter and the conserved Ets DNA binding domain along is sufficient to mediate the interaction between Ets-2 and p300/CBP *in vitro* (36, 37). Since we previously showed that the binding of E1A to p300/CBP and the binding motif of p300/CBP on Her-2/neu promoter are crucial for the ability of E1A to inhibit Her-2/neu promoter activity (38), it is worth investigating whether p300/CBP plays a similar role in PEA3-mediated inhibition of Her-2/neu transcription. If this is the case, it may provide another complementary mechanism for the transcription repression of Her-2/neu by PEA3.

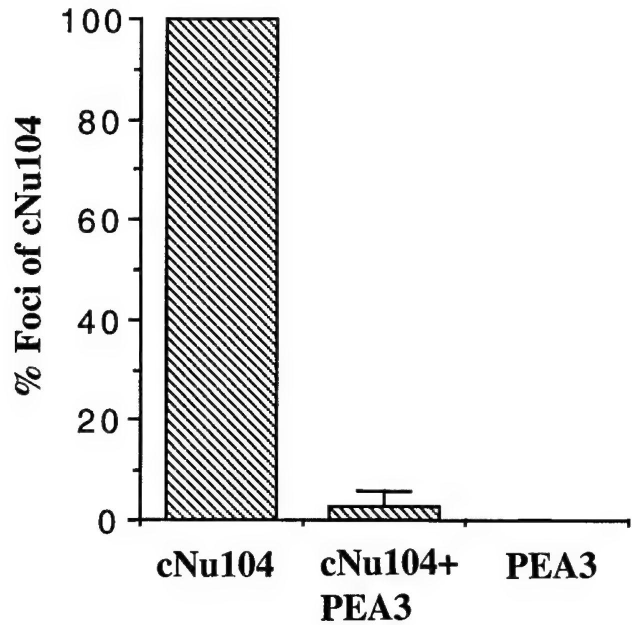
(2). Identify the Ets family member(s) that binds to the Ets consensus motif and activates transcription of Her-2/neu.

Although several candidate Ets members have been suggested to possibly involve in regulation of Her-2/neu transcription (39), it still remains unclear which Ets family member(s) is responsible for the positive regulation of Her-2/neu transcription. To make our competition model complete, we hope to identify the Ets family member(s) regulating Her-2/neu transcription and further confirm our hypothesis.

(3). Develop DNA binding domain of PEA3 (PEA3 DBD) as a potential gene therapy agent for breast cancer and ovarian cancer with Her-2/neu overexpression.

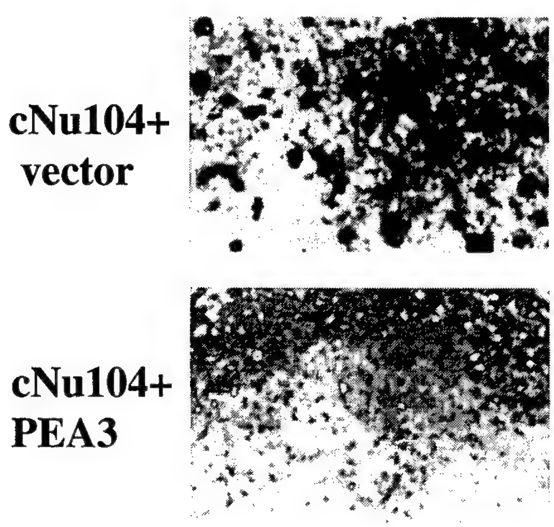
Many PEA3 target genes have been reported and a significant fraction of these genes encode proteases required for degradation of the extracellular matrix (40, 41, 42, 43). The unregulated expression of these enzymes is associated with metastatic propensity of tumor cells (44). Therefore, one withdraw to use PEA3 as a gene therapy agent is that the metastatic potential of cancer cells may possibly be enhanced. In this study we also showed that DNA binding domain of PEA3 (PEA3 DBD) along is sufficient to repress Her-2/neu transcription. Presumably, PEA3 DBD does not have this problem in contrast to full length PEA3 as a gene therapy agent for breast cancer and ovarian cancer overexpressing Her-2/neu. Besides, PEA3 DBD may also be able to repress the transcription of PEA3 target genes encoding protease via a dominant-negative route and thus suppress the metastatic potential of cancer cells. It would be intriguing and worthwhile to test the feasibility of developing PEA3 DBD as a potential gene therapy agent for breast cancer and ovarian cancer overexpressing Her-2/neu.

A.



100
80
60
40
20
0
% Foci of cNu104
cNu104
cNu104+
PEA3
PEA3

B.



cNu104+
vector
cNu104+
PEA3

Fig.1 A&B

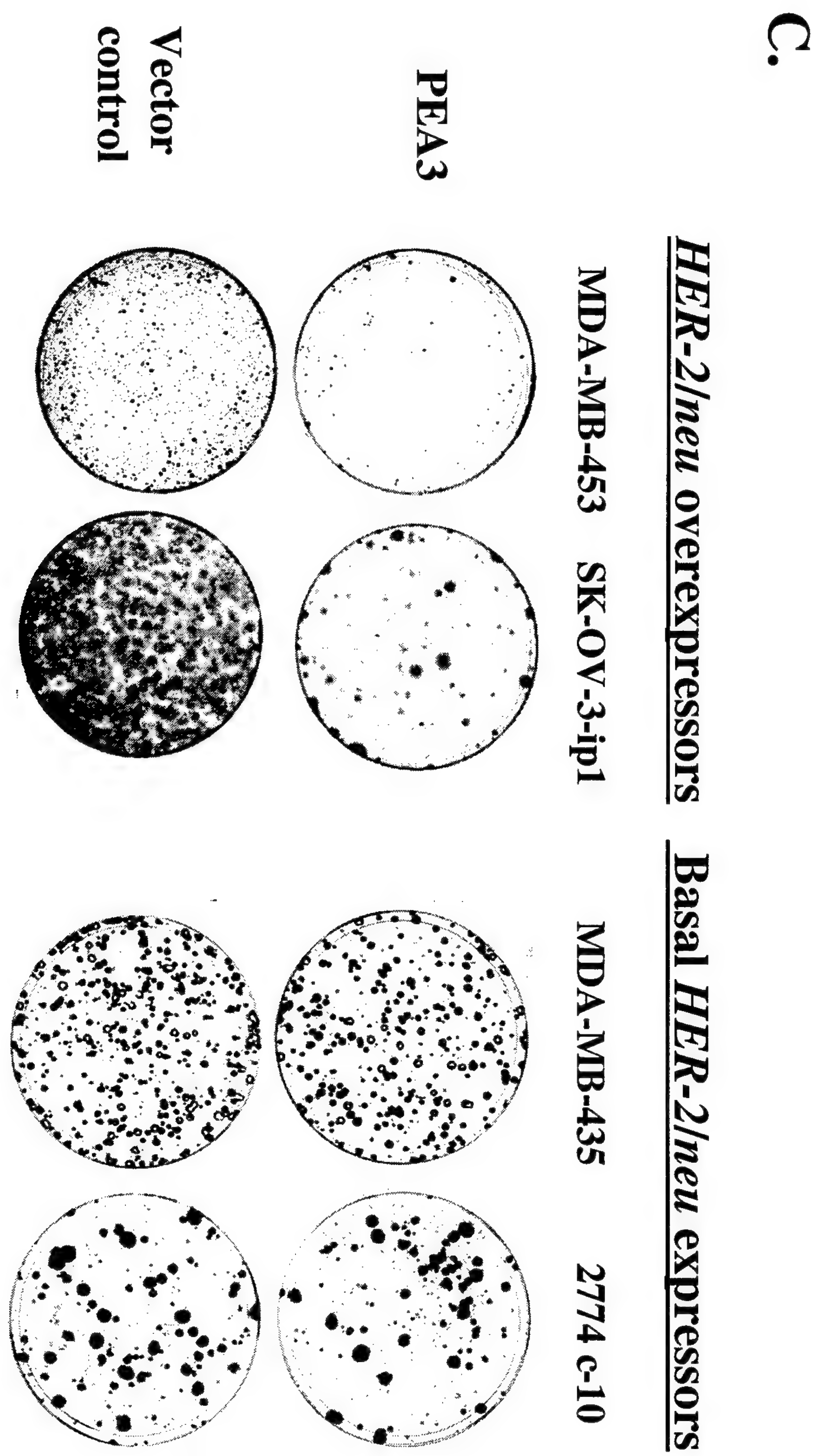

C.
HER-2/neu overexpressors
Basal HER-2/neu expressors
MDA-MB-453
SK-OV-3-ip1
MDA-MB-435
2774 c-10
PEA3
Vector control

Fig.1 C

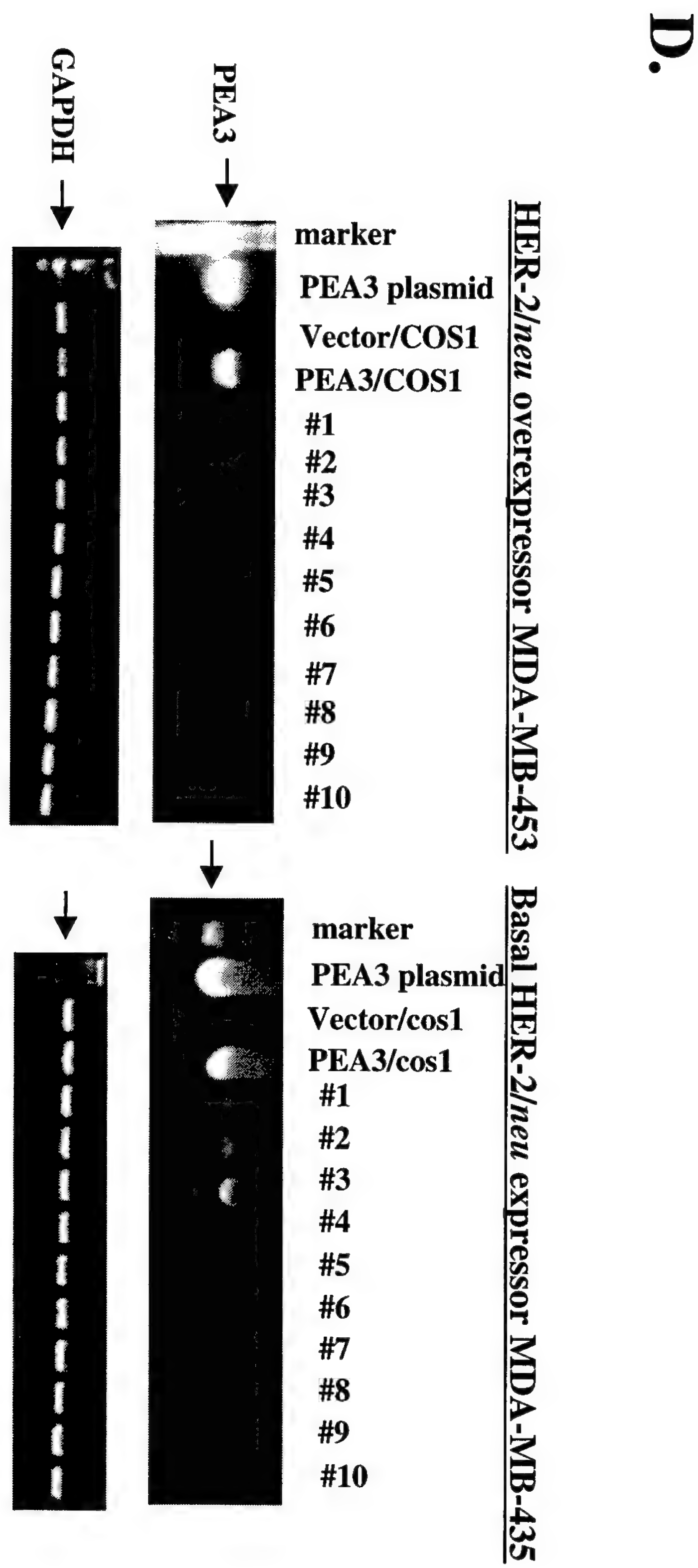

D.
HER-2/neu overexpressor MDA-MB-453
marker
PEA3 plasmid
Vector/COS1
PEA3/COS1
#1
#2
#3
#4
#5
#6
#7
#8
#9
#10
PEA3
GAPDH
Basal HER-2/neu expressor MDA-MB-435
marker
PEA3 plasmid
Vector/cos1
PEA3/cos1
#1
#2
#3
#4
#5
#6
#7
#8
#9
#10

Fig.1 D

Figure 1. PEA3 represses neu-mediated transformation and inhibits cell growth of HER-2/neu-overexpressing cancer cells.

A. PEA3 represses the transforming activity of activated genomic rat neu. NIH3T3 cells ($2x10^5$) were plated to a 100-mm culture dish 24 hours before transfection. Cells were transfected with 0.5 μg of pSV2neo, 1 μg of cNu104 plus 5 μg of either pSRαPEA3 or the control vector pSV2E by calcium phosphate precipitation. Two days after transfection, cells were split and two plates were subject to selection in 500 μg/ml of neomycin (G418; Life Technologies, Inc), and the other two were maintained in normal medium until foci appeared. Foci and neomycin-resistant colonies were stained and counted. The number of foci was normalized by the corresponding number of neomycin-resistant colonies. Results are expressed as ratio of the number of foci to the number of colonies from each transfection. The ratio of cNul04 transfection alone was set as 100 %.

B. An example of the PEA3-mediated suppression of the focus forming activity by cNul04.

C. Cell growth of HER-2/neu overexpressing breast and ovarian cancer cells was inhibited by PEA3 transfection as indicated by the decrease of colony numbers compared with vector trasfection.

D. Ectopic PEA3 RNA expression was preferentially lost in the survival neomycin-resistant clones derived from the HER-2/neu-overexpressing breast cancer cell line MDA-MB453. Total RNA was extracted from cell clones resulted from stable transfection and RT-PCR was performed by following the manufacturer's instruction (SuperScript preamplification system; Life Technologies, Inc). The primers derived from the PEA3 coding sequence (5'-TGAATTATGACAAGCTGAGCCG-3') and from the expression vector pcDNA3 (5'-TCAGCGAGCTCTAGCATTTAGG-3') were use to amplified the ectopically expressed PEA3 transcript. Primers for GAPDH internal control were: 5'AGGTGAAGGTCGGAGTCAAC-3' and 5'-TCCATTGATGACAAGCTTCCC-3'. COS-1 cells transiently transfected by the PEA3 cDNA were used as a positive control. Amplification was performed on a Perkin Elmer DNA Cycler 480 for 35 cycles with denaturing at 94°C for 30 seconds, annealing at 58°C for 1.5 minutes and extension at 72°C for 1.5 minutes.

Table I. PEA3 suppresses growth of cancer cells overexpressing HER-2/neu.[1]

HER-2/neu overexpressers

Cell lines	pcDNA3	PEA3
SK-BR-3	100	0.7 (±0.9)
MDA-MB-453	100	19.7 (±3.0)
SKOV3-ip1	100	14.3 (±4.1)

HER-2/neu basal-level expressers

Cell lines	pcDNA3	PEA3
MDA-MB-435	100	73.4 (±10.9)
2774 c-10	100	83.3 (±3.8)

[1] Experiment for each cell line was independently repeated 2-4 times using different batches of plasmid DNA and cell culture. Inhibition of colony formation by PEA3 transfection is shown in percentage with the number of neomycin-resistant colony resulted from pcDNA3 transfection arbitrarily set as 100 %.

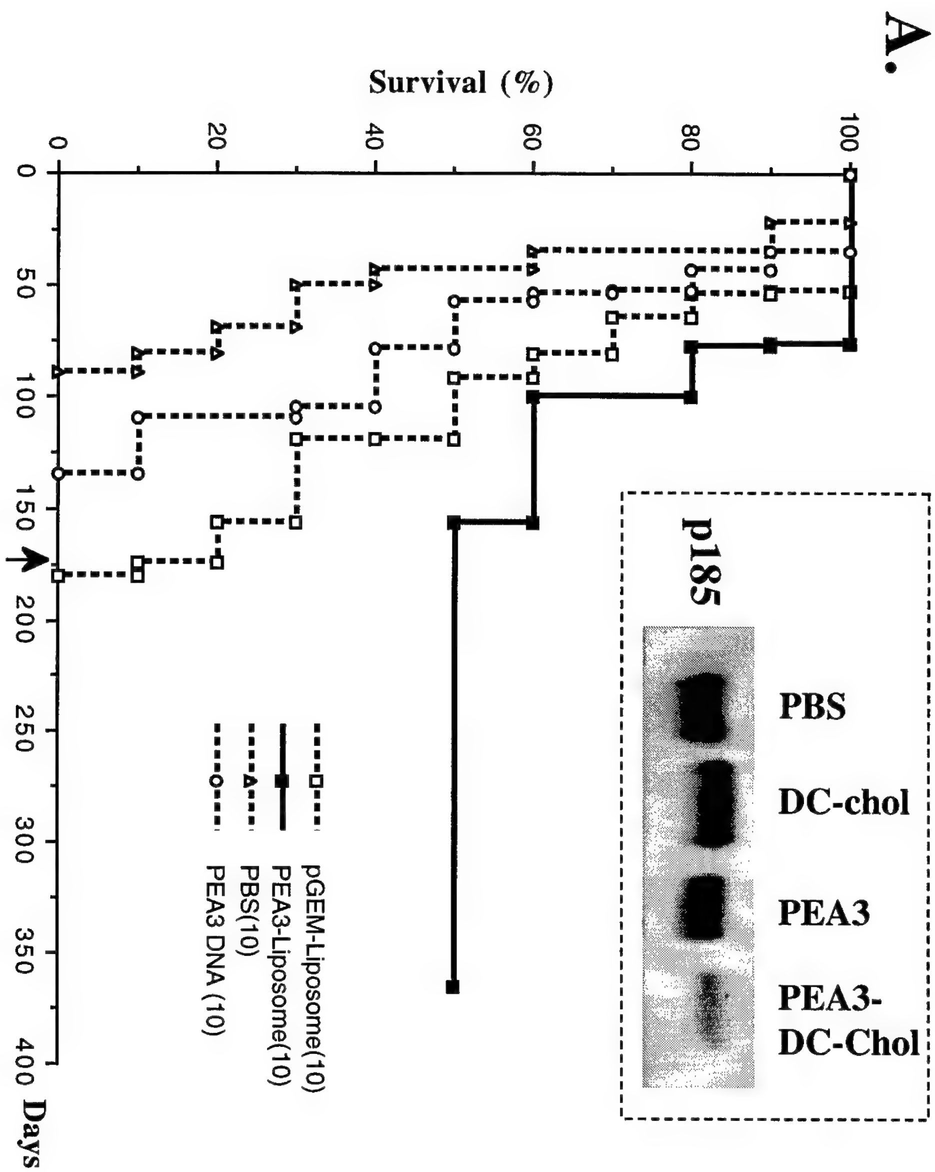

A.
Survival (%)
0
20
40
60
80
100
0
50
100
150
200
250
300
350
400
Days
p185
PBS
DC-chol
PEA3
PEA3-
DC-Chol
pGEM-Liposome(10)
PEA3-Liposome(10)
PBS(10)
PEA3 DNA (10)

Fig. 2A

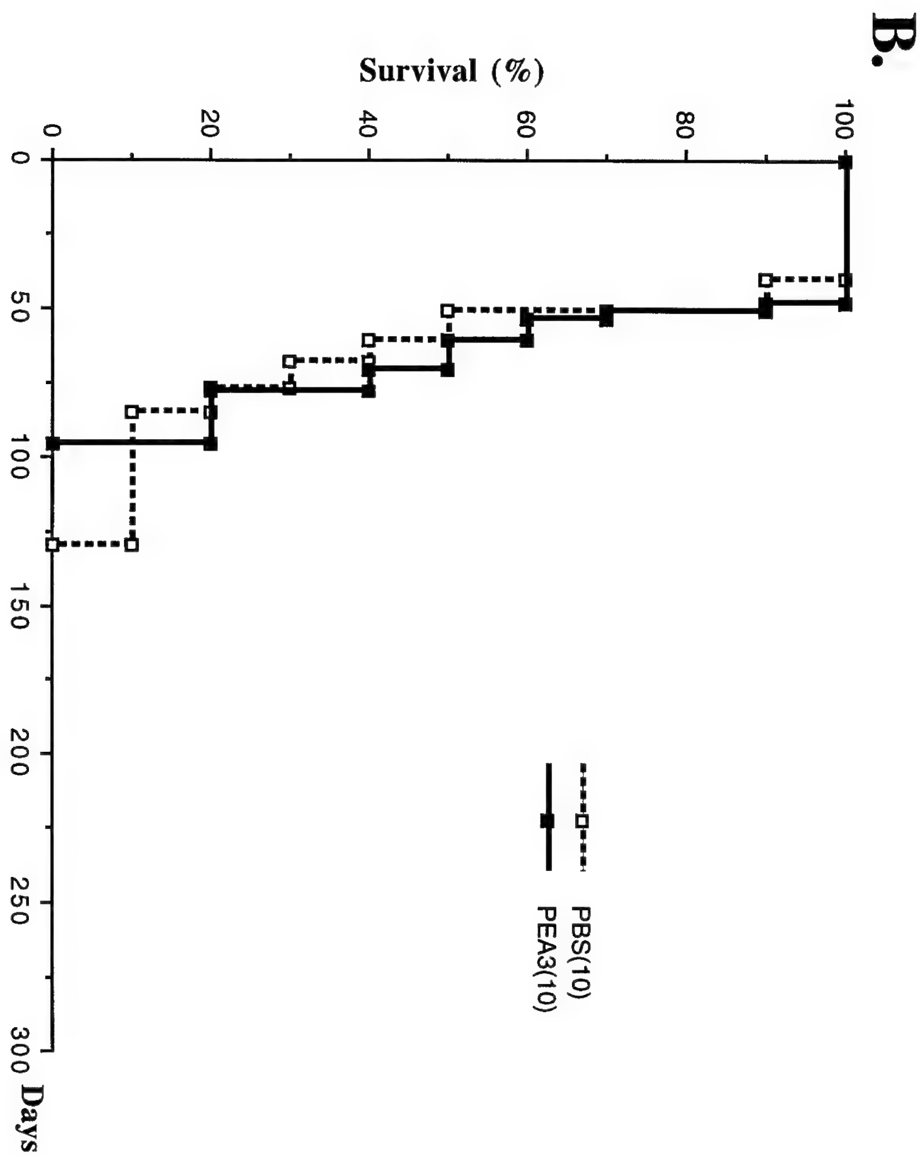

B.
Survival (%)
100
80
60
40
20
0
0
50
100
150
200
250
300
Days
PBS(10)
PEA3(10)

Fig. 2B

Figure 2. PEA3 inhibits growth of tumor xenografts derived from HER-2/neu overexpressing cancer cells.

A. Nude mice carrying intraperitoneal tumors derived from SKOV3-ip1 received weekly i.p. injections of a reagent containing PEA3 plasmid DNA complexed with liposome (PEA3-liposome), control DNA (pGEM) complexed with liposome (pGEM-Liposome), naked PEA3 plasmid DNA, or PBS. Number of mice in each group is shown in the parentheses. The arrow marks the last injection. Tumor samples were retrieved from mice treated with PBS, the lipid vehicle DC-Chol, PEA3 naked DNA, or PEA3-DC-Chol complex, and the levels of p 185 were measured by immunoblotting as shown in the insert. Loading was controlled by the endogenous α-actin expression (data not shown).

B. Nude mice carrying tumors derived from 2774 c-10 were treated with PEA3-liposome complex (the solid line) or PBS alone (the dashed line) by the same procedure as SKOV3-ipl cells.

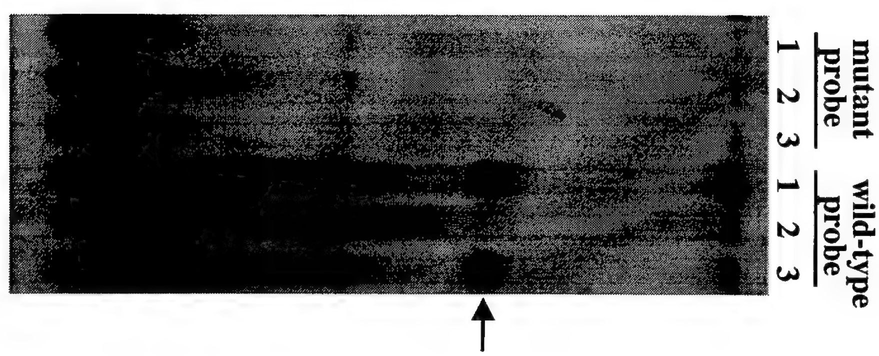
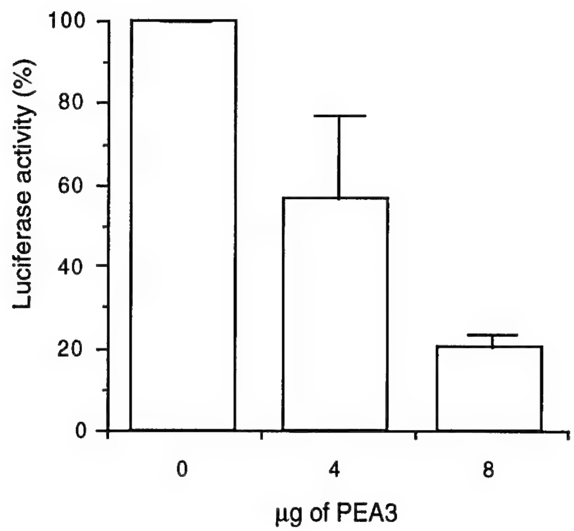


Fig. 3

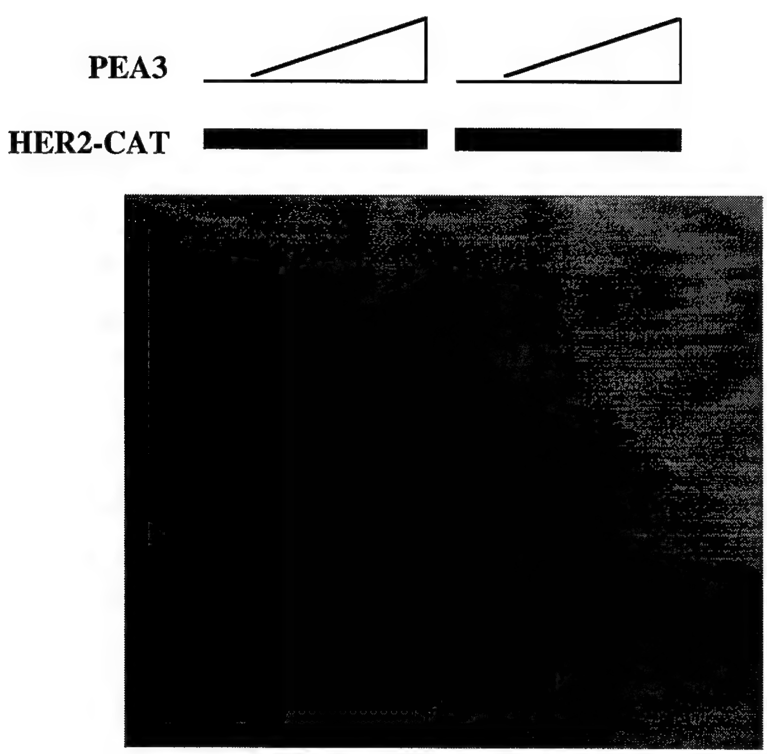
Figure 3. PEA3 binds to the HER-2/neu promoter. GST-PEA3 fusion protein was incubated with ^{32}p-γ ATP end-labeled wild-type or mutated oligonucleotide, containing the consensus PEA3 binding site sequence on the HER-2/neu promoter (lanes 1). The binding specificity was tested by competition with unlabeled wild-type (lanes 2) or nonspecific oligonucleotide (lanes 3).

A.



Luciferase activity (%)
100
80
60
40
20
0
0
4
8
µg of PEA3

B.

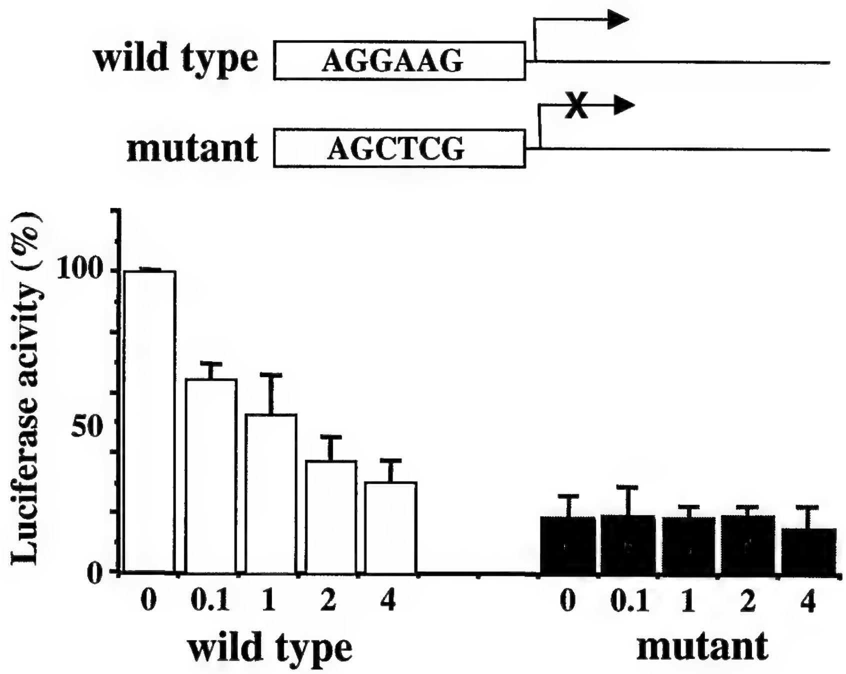


PEA3
HER2-CAT

Fig. 4

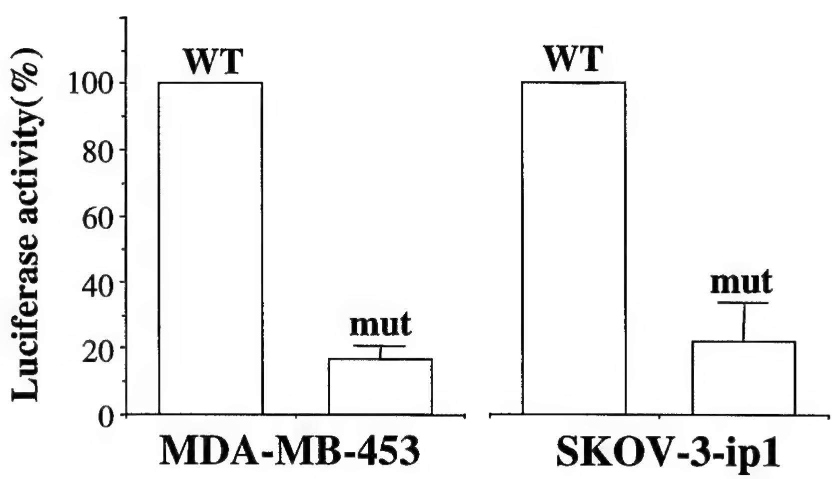
Figure 4. PEA3 inhibits the HER-2/neu promoter activity in HER-2/neu overexpressing cancer cell lines. Human ovarian cancer cells SKOV-3 were cotransfected with 5 μg of pNeulit or HER2-CAT with different amounts of PEA3 plasmid DNA (pcDNA3-PEA3) as indicated. The luciferase (**A**) or the chlroarnphenicol acetyltransferase (CAT) (**B**) reproter activity was measured. For the CAT transient assay, the doses of cotransfected pcDNA3-PEA3 were 0, 5, 10, and 15 μg. The results of two independent experiments are shown.

A.



wild type
AGGAAG
mutant
AGCTCG
X
Luciferase acivity (%)
100
50
0
0
0.1
1
2
4
wild type
0
0.1
1
2
4
mutant

B.



Luciferase activity(%)
100
80
60
40
20
0
WT
mut
WT
mut
MDA-MB-453
SKOV-3-ip1

Fig. 5

Figure 5. PEA3 represses HER-2/neu promoter activity through a positive regulatory DNA motif.

A. Luciferase reporter gene driven by either wild-type (open bars) or the PEA3 site-mutated (close bars) HER-2/neu promoter was measured after co-transfection with PEA3 cDNA into MDA-NM-453 cells.

B. The luciferase activities derived from wild-type and the PEA3 site-mutated HER-2/neu promoter were compared by transfection into a breast cancer cell line (MDA-MB-453) and a ovarian cancer cell line (SKOV-3-ip1).

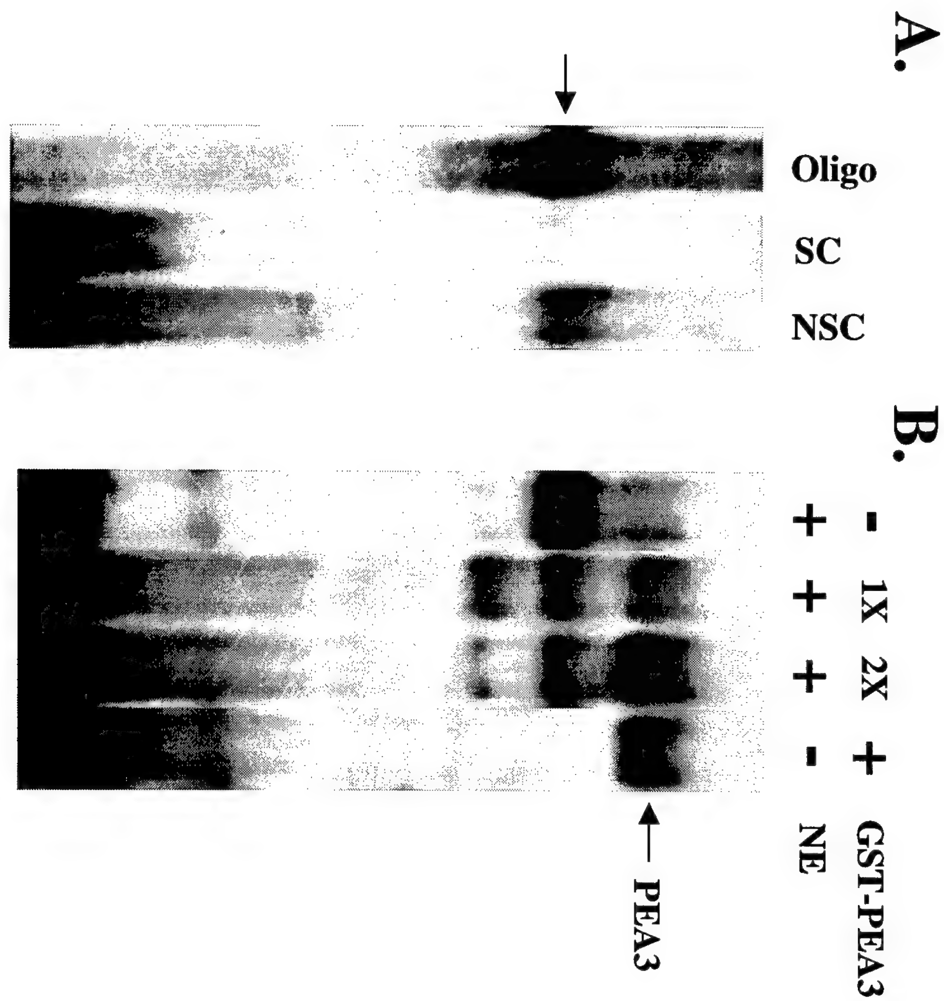


Fig. 6A&B

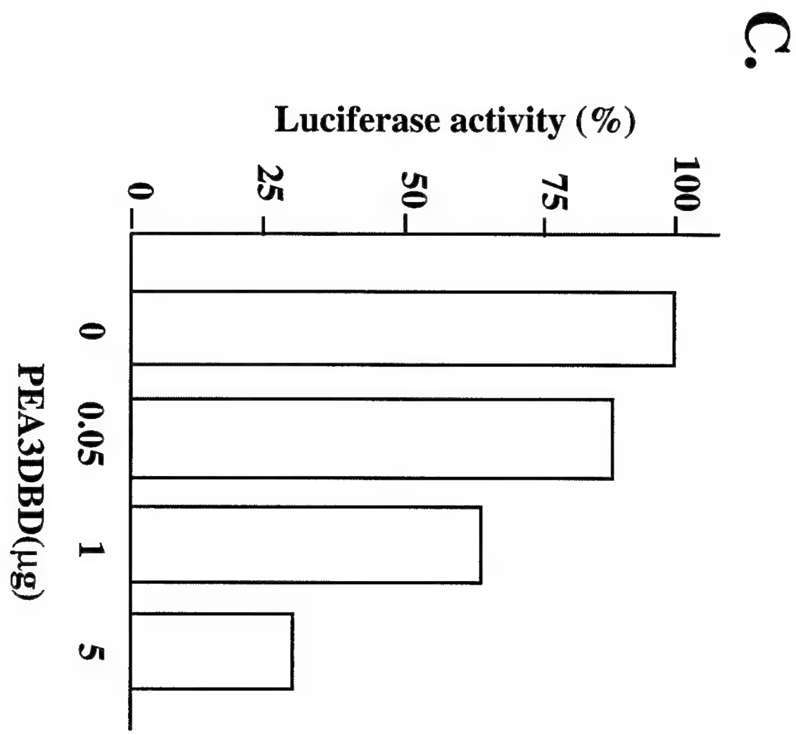


Fig. 6C

Figure 6. PEA3 competes with a distinct family member of the Ets family detected in HER-2/neu-overexpressing cancer cells.

A. Nuclear extract of MDA-MB-453 cells was incubated with the DNA oligonucleotide probe in the absence (Oligo) or presence of unlabeled competitor of the same specific oligonucleotide (SQ or non-specific oligonucletide of shuffled sequences (NSC). The signal of specific DNA-protein complex is indicated by an arrow.

B. Increasing amount of GST-PEA3 fusion protein was added into the binding reaction to test the competition between the unknown nuclear factor and PEA3 protein. The DNA-PEA3 complex is indicated by an arrow. NE, nuclear extract.

C. The DNA-binding domain of PEA3 is sufficient to down-regulate the HER-2/neu promoter as assayed by cotransfection of different doses of PEA3DBD and pNulite. The result from a representative experiment is shown.

KEY RESEARCH ACCOMPLISHMENTS

A. Explored and confirmed the feasibility of development of PEA3 as a potent gene therapy agent for breast cancer and ovarian cancer overexpressing Her-2/neu.

(1). PEA3 suppress the HER-2/neu transforming phenotype in vitro.

(2). PEA3 inhibits tumor growth in vivo.

B. Provided a competition model to elucidate the molecular mechanism for the transcription inhibition of Her-2/neu by PEA3.

(1). PEA3 binds to the HER-2/neu promoter.

(2). PEA3 represses the HER-2/neu promoter activity through a positive regulatory motif of the promoter; competition of PEA3 with other Ets transactivating factor(s) may account for the down-regulation of Her-2/neu by PEA3.

REPORTABLE OUTCOMES

(1). Results achieved in this study have been submitted to the journal of *Nature Medicine* (manuscript appended).

Xiangming Xing, Shao Chun Wang, Susan Miller, Leaf Huang, Wei Ya Xia, and **Mien-Chie Hung**. The Ets Protein PEA3 Suppresses HER-2/Neu Overexpression And Inhibits Tumorigenesis

(2). Supported by this award, Ms. Xiangming Xing obtained her Ph.D. degree from the Graduate School of Biomedical Sciences The University of Texas-Houston.

(3). Part of the graduate training of Mr. Zhenming Yu is supported by this award.

CONCLUSION

In this study, we explored and confirmed the feasibility of development of PEA3 as a potent gene therapy agent for breast cancer and ovarian cancer overexpressing Her-2/neu. PEA3 suppresses the HER-2/neu transforming phenotype in vitro and inhibits tumor growth in vivo. A competition model to elucidate the molecular mechanism for the transcription inhibition of Her-2/neu by PEA3 was proposed. PEA3 directly binds to the HER-2/neu promoter and competition of PEA3 with other Ets transactivating factor(s) for a positive regulatory motif on the Her-2/neu promoter may account for the down-regulation of Her-2/neu transcription by PEA3.

REFERENCE

1. Boring, C.C., Squires., T.S., Tong, T., and Montgomery, S. *CA-Cancer J. Clin.*, 44:7-27 (1994)
2. Piver, M., Baker, T., Piedmonte, M., Sandecki, A. *Seminars in Oncology* 18(3):177-185 (1991)
3. Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A., and McGuire, W. L. *Science* 240,177-182 (1987)
4. McCann, A. H., Dervan, P. A., O'Regan, M., Codd, M. B., Gullick, W. J., Tobin, B. M. J., Carney, D. N.. *Cancer Res.* 51: 3296-3303 (1991)
5. Vijver, M. R. Bersselaar, P. Devilee, C. Cornelisse, J. Peterse, and R. Nusse. *Mol. Cell. Biol.* 7:2019-2023 (1987).
6. Gusterson, B., Gelber, R., Goldhirsch, A., Price, K., Save-Soderborgh, J., Anbazhagan, R., Styles, J., Rudenstam, C-M, Golouh, R., Reed, R., MartinezTello, F., Tiltman, A., Torhorst, J., Grigolato, P., Bettelheim, R., Neville, A., Burki, K., Castiglione, M., Collins, J., Lindtner, J., and Senn, H-J. *J. Clin. Onc.* 10(7):10491056 (1992)
7. Toikkanen, S., Helin, H., Isola, J., and Joensuu, H. *J. Clin. Onc.* 10(7):1044-1048 (1992)
8. Slamon, D.J., G. Williams, L.A. Jones, J.A. Holt, S.G. Wong, D.E. Keith, W.J. Levin, S.G. Stuart, J. Udove, A. Ullrich, and M.F. Press. *Science* 244:707712 (1989)
9. Yusa, K., Y. Sugirnot, T. Yarnori, T. Yamamoto, K. Toyoshima and T. Tsuruo. *J. Natl. Cancer Inst.* 82:1633-1636 (1990)
10. Tsai, C-M., Yu, D., Chang, K-T., Wu, L-H., Perng, R-P. *J. Nad. Cancer Inst.* 87:682-684, (1995)
11. Tsai, C-M., Chang, K-T., Perng, R-P., Mitsudomi, T., Chen, M-H. *J. Natl. Can Inst.* 85(11): 897-901, (1993)
12. Yu, D. & Hung, M.-C. *Cancer Metastasis Rev.* 17, 195-202 (1998).
13. Yu, D., Suen, T.C., Yan, D.H., Chang, L.S. & Hung, M.-C. *Proc. Natl. Acad. Sci. USA* 87, 4499-4503 (1990).

14. Yu, D., Hamada, J., Zhang, H., Nicolson, G.L. & Hung, M.-C. *Oncogene* 7, 2263-2270 (1992).
15. Yu, D., Suen, T.C., Yan, D.H., Chang, L.S. & Hung, M.-C. *Oncogene* 7, 2263-2270 (1992).
16. Zhang, Y., Yu, D., Xia, W. & Hung, M.-C. *Oncogene* 10, 1947-1954 (1995).
17. Xing, X. *et al Cancer Gene Ther.* 3, 168-174 (1996).
18. Hung, M.-C., Schechter, A.L., Chevray, P.L., Stern, D.F. & Weinberg, R.A. *Proc. Natl. Acad. Sci. USA* 83, 261-264 (1986).
19. Bargmann, C.I., Hung, M.-C. & Weinberg, R.A. *Cell* 45, 649-657 (1986).
20. Yu, D.H., Scorsone, K. & Hung, M.-C. *Mol. Cell. Biol.* 11, 1745-1750 (1991).
21. Yu, D. *et al. Oncogene* 11, 1383-1388 (1995).
22. White, M.R. & Hung, M.-C. *Oncogene* 7, 677-683 (1992).
23. Buttice, G. & Kurkinen, M. *J. Biol. Chem.* 268, 7196-7204 (1993).
24. Zhang, M., Magit, D. & Sager, R. *Proc. Natl. Acad. Sci. USA* 94, 5673-5678 (1997).
25. Zhang, M., Maass, N., Magit, D. & Sager, R. *Cell Growth Differ.* 8, 179-186 (1997).
26. Sgouras, D.N. *et al. EMBO J.* 14, 4781-4793 (1995).
27. Giovane, A., Pintzas, A., Maira, S.M., Sobieszczuk, P. & Wasylyk, B. *Genes Dev.* 8, 1502-1513 (1994).
28. Chen, H.M. & Boxer, L.M. *Mol. Cell. Biol.* 15, 3840-3847 (1995).
29. Goldberg, Y., Treier, M., Ghysdael, J. & Bohmann, D. *J. Biol. Chem.* 269, 16566-16573 (1994).
30. Hung, M.-C. *et al. Gene* 159, 65-71 (1995).
31. Xin, J.H., Cowie, A., Lachance, P. & Hassell, J.A. *Genes Dev.* 6, 481-496 (1992).
32. Baselga, J., Norton, L., Albanell, J., Kim, Y.M. & Mendelsohn, J. *Cancer Res.* 58, 2825-2831 (1998).
33. Pegram, M.D. *et al. J. Clin. Oncol.* 16, 2659-2671 (1998).
34. Chang, J.Y. *et al. Oncogene* 14, 561-568 (1997).
35. Hung, M.-C., Wang, S.-C. & Hortobagyi, G. Targeting *HER-2/neu*-overexpressing cancer cells with transcriptional repressor genes delivered by cationic liposome. in *Non-viral vectors for gene therapy* (eds. Huang, L., Hung, M.-C. & Wagner, E.) (Academic Press, San Diego, CA, 1999, in press).

36. Yang C, Shapiro LH, Rivera M, Kumar A, Brindle PK. *Mol Cell Biol* 18(4):2218-29 (1998)

37. Jayaraman G, Srinivas R, Duggan C, Ferreira E, Swaminathan S, Somasundaram K, Williams J, Hauser C, Kurkinen M, Dhar R, Weitzman S, Buttice G, Thimmapaya B. *J Biol Chem* 274(24):17342-52 (1999)

38. Chen, H. & Hung, M.-C. *J. Biol. Chem.* 272, 6101-6104 (1997).

39. Scott GK, Daniel JC, Xiong X, Maki RA, Kabat D, Benz CC, *J Biol Chem* 269(31):19848-58 (1994)

40. Rorth P, Nerlov C, Blasi F, Johnsen M, *Nucleic Acids Res* 18(17):5009-17 (1990)

41. Gutman A, Wasylyk BAuble DT, Brinckerhoff CE, *EMBO J.* 9(7):2241-6 (1990)

42. Wilson CL, Matrisian LM, *Int J Biochem Cell Biol* 28(2):123-36 (1996)

43. Crawford HC, Matrisian LM, *Enzyme Protein* 49(1-3):20-37 (1996)

44. Kohn EC, Liotta LA, *Cancer Res.* 55(9):1856-62 (1995)

APPENDICES

1. **A manuscript of the paper describing the result of this study is appended. This paper was submitted to the journal of *Nature Medicine*.**

   Xiangming Xing, Shao Chun Wang, Susan Miller, Leaf Huang, Wei Ya Xia, and **Mien-Chie Hung**. The Ets Protein PEA3 Suppresses HER-2/Neu Overexpression And Inhibits Tumorigenesis

2. **Personnel receiving pay from this research effort:**

   Xiangming Xing
   Zhenming Yu

The Ets Protein PEA3 Suppresses HER-2/Neu Overexpression And Inhibits Tumorigenesis

Xiangming Xing[1‡], Shao Chun Wang[1‡], Susan Miller[1], Leaf Huang[2], Wei Ya Xia[1], and Mien-Chie Hung[1*]
University of Texas M. D. Anderson Cancer Center Department of Cancer Biology, Section of Molecular Cell Biology, Houston, Texas, 77030[1]; Department of Pharmacology, University of Pittsburg, Pittsburg, Pennsylvania 15261[2].

‡These authors equally contributed to the work.
* To whom correspondence should be addressed. Phone: (713) 792-3668. Fax: (713) 794-4784. E-mail: mchung@odin.mdacc.tmc.edu
Key Words: PEA3, HER-2/neu overexpression, tumorigenecity, transformation

HER-2/neu overexpression plays an important role for cancer development. To suppress cell transformation mediated by HER-2/neu overexpression, we identified a DNA-binding protein PEA3 of the *ets* gene family which specifically targeted a DNA sequence on the HER-2/neu promoter and downregulated the promoter activity. Expression of PEA3 resulted in preferential inhibition of cell growth and tumor development of HER-2/neu-overexpressing cancer cells. This study demonstrates a novel approach targeting HER-2/neu overexpression and also provides a general and promising approach to design repressors for diseases caused by overexpression of pathogenic genes.

INTRODUCTION

Overexpression of the HER-2/neu gene (also known as c-erbB2) is frequently associated with many types of cancers including breast and ovarian cancers [1-7]. The overall survival rate of breast and ovarian cancer patients whose tumors have HER-2/neu overexpression is significantly lower than that of patients whose tumors do not have HER-2/neu overexpression [8-11]. A large body of evidence indicates that HER-2/neu overexpression is associated with elevated tumorigenecity [12], enhanced metastatic potential [13-17], and, in certain circumstances, increased resistance to chemotherapy [18-22].

On the other hand, downregulation of the HER-2/neu oncogene confers suppression to the transforming phenotype induced by the oncogene [12, 23-27]. We have shown previously that the adenovirus type 5 E1A gene can indirectly downregulate HER-2/neu promoter activity through the coactivator p300 [28]. Downregulation of HER-2/neu overexpression by E1A significantly mitigated tumorigenic activity of human breast and ovarian cancer cells in nude mice [12, 26, 29, 30]. These results strongly implicate the potential of inhibiting HER-2/neu-mediated cell transformation by transcriptional repressors which target the promoter of the oncogene.

One strategy to efficiently suppress oncogene-mediated transformation is to inhibit the activity of oncogene promoter by DNA-binding protein that can directly bind to specific DNA sequence on the promoter and result in inhibition of transcription activity. A DNA motif (5'AGGAAG3') containing the consensus binding site of PEA3 [31], a member of the *ets* transcription factor family, is present 26 nucleotides upstream from the major transcriptional start site on the HER-2/neu promoter [32]. The sequence and position of the motif is conserved among human, rat, and mouse HER-2/neu promoters [33]. In this study, we show evidence that PEA3 directly binds to the consensus motif and prevents HER-2/neu gene overexpression by suppressing the promoter activity. Down-regulation of HER-2/neu expression resulted in reversing the transforming phenotype

induced by HER-2/neu activation, inhibiting cell growth *in vitro*, and, in a preclinical gene therapy setting, blocking tumor formation from HER-2/neu-overexpressing cancer cells and prolonging survival rate of treated animals. Our study demonstrates a promising approach to design repressors targeting diseases caused by overexpression of pathogenic genes.

RESULTS

PEA3 binds to the HER-2/neu promoter

A DNA motif with the sequence of 5'AGGAAG3' has been identified on the HER-2/neu promoter [33]. To test whether PEA3 can recognize and bind to this putative PEA3 binding site, purified GST-PEA3 fusion protein was prepared and incubated with ^{32}P-labeled oligonucleotide probes containing either the wild-type PEA3 binding site sequence or the same sequence but with the core PEA3 binding motif mutated (5'AGCTCG3'). DNA-protein association was investigated with an electrophoretic mobility shift assay (EMSA). Specific binding between the fusion protein and the wild-type probe was identified (Fig. 1). The binding was diminished in the presence of unlabeled wild-type oligonucleotide. There was no detectable association between the fusion protein and the mutant oligonucleotide. GST alone does not bind to the probe (data not shown).

PEA3 represses the HER-2/neu promoter activity through a positive regulatory motif of the promoter

To investigate the effect of PEA3 to the promoter activity of HER-2/neu, a luciferase reporter gene driven by the HER-2/neu promoter (pNulit) was cotransfected with different amount of PEA3 cDNA into the SKOV-3 ovarian cancer cell line (Fig. 2A). The PEA3 cDNA repressed the promoter activity of HER-2/neu in a dose-dependent manner. Similar results were observed in experiments using a breast cancer cell line MDA-MB-453. In these experiments both the luciferase (Fig. 2A, 3A) and the chloramphenical acetyl transferase (HER2-CAT; Fig. 2B) reporter genes consistently demonstrated that PEA3 coexpression can repress the HER-2/neu promoter activity. These results indicate that PEA3 is a potent transrepressor of HER-2/neu gene expression. The intact PEA3 binding site on the HER-2/neu promoter is required for the

PEA3-mediated transcriptional repression because the HER-2/neu promoter with a mutated PEA3 binding sequence (5'AGGAAG3' to 5'AGCTCG3') was not subject to the negative regulation by PEA3 (Fig. 3A). The results suggest that PEA3-mediated HER-2/neu downregulation is through the PEA3 binding site on the HER-2/neu promoter. When the promoter activities were compared between the wild-type and mutant promoters in the absence of PEA3, the activity of the mutant promoter was significantly lower than that of the wild-type promoter (Fig. 3B), indicating that the PEA3 binding site on the HER-2/neu promoter actually acts as a positive regulatory element for elevated expression of HER-2/neu.

PEA3 suppress the HER-2/neu transforming phenotype *in vitro*

To test whether expression of PEA3 can repress HER-2/neu expression and therefore reverse the transforming phenotype, a genomic activated rat neu oncogene (cNu104) that was known to transform mouse fibroblast cells and result in enhanced focus-forming activity [34-36] was subject to a focus forming assay by cotransfecting with the PEA3 cDNA or the control vector into the mouse fibroblast NIH3T3 cells. The results indicated that PEA3 dramatically suppressed the focus formation caused by cNu104 (Fig. 4, A and B).

To test if ectopic expression of PEA3 can suppress cell growth of cancer cells, breast and ovarian cancer cells with high or basal level HER-2/neu expression were transfected with a plasmid carrying a neomycin resistance gene and the PEA3 cDNA controlled by a CMV promoter. Subsequent neomycin selection resulted in numerous resistant colonies from the HER-2/neu low expressing cell lines while only few colonies of cell lines with HER-2/neu overexpression were retained through the selection (Fig. 4C and Table I). The surviving colonies were isolated and tested for ectopic expression of PEA3 by RT-PCR. Virtually all of the clones derived from the HER-2/neu low expressor cell line (MDA-MB-435), while only two clones derived from the HER-2/neu

overexpressing cell line (MDA-MB-453), contained PEA3 RNA expressed from the plasmid (Fig. 4D). One of the two clones from MDA-MB-453 (clone No. 2) did not express detectable PEA3 protein. Another clone (clone No. 8) expressing ectopic PEA3 protein grew extremely slow and eventually lost during subsequent cell culturing (data not shown). The facts that transfection of PEA3 gene into HER-2/neu-overexpressing cells reduces the number of neomycin-resistant colonies and that the survived clones either do not express PEA3 protein or is unable to grow in cell culture strongly suggest that PEA3 inhibits growth of HER-2/neu-overexpressing cancer cells.

PEA3 inhibits tumor growth *in vivo*

To further investigate the tumor suppression potential of PEA3, we used an established animal model to test whether PEA3 could suppress tumor development in animals and prolong animal survival [27, 29]. Ovarian cancer orthotopic tumor xenografts derived from a HER-2/neu-overexpressing cell line (SKOV3-ip1) or a cell line with only basal level of HER-2/neu expression (2774 c-10) were developed intraperitoneally in nude mice. Tumor-bearing mice were treated with or without PEA3-expressing plasmid DNA once per week delivered by a cationic liposome (DC-Chol) by intraperitoneal injection. Fifty percent of mice with SKOV3-ip1 tumors survived without detectable tumors for longer than one year after treated by PEA3 plus liposome, while all mice in the control groups died within half year (Fig. 5A). On the other hand, no response to PEA3-liposome treatment was observed for mice bearing 2774 10-c-derived tumors (Fig. 5B). The therapeutic effect of PEA3 combined with the cationic liposome was associated with downregulation of the HER-2/neu p185 protein product in the treated tumors of SKOV3-ip1 xenograft (Fig. 5A insert).

PEA3 competes with other transactivating factor(s)

The results shown above demonstrate that PEA3 down-regulates HER-2/neu

expression and suppresses the transforming phenotype mediated by HER-2/neu overexpression. The fact that the PEA3 binding motif on the HER-2/neu promoter functions as a positive regulatory element for HER-2/neu gene transcription (Fig. 3B) implicates the presence of a yet unidentified transactivating factor(s) which can recognize the same positive regulatory element and that PEA3 may compete with the transactivating factor(s) for the same DNA motif and result in repression of HER-2/neu transcription. To test this hypothesis, nuclear extract from the HER-2/neu-overexpressing cell line MDA-MB-453 was prepared and tested for binding activity to a oligonucleotide probe containing DNA sequence of the PEA3 binding site in the presence of increasing amount of GST-PEA3 fusion protein. The EMSA results revealed a specific nuclear binding activity to the PEA3 binding site (Fig. 6A). The binding of the unknown factor(s) is unique and can be distinguished from the PEA3-DNA complex. Furthermore, increasing doses of PEA3 fusion protein diminished the unique binding activity in MDA-MB-453 cells (Fig. 6B), indicating a competition relationship between PEA3 and the unidentified transcriptional factor(s). This competition model suggests that overexpression of the DNA-binding activity of PEA3 would be sufficient to suppress HER-2/neu gene expression by blocking the binding of other transactivators to the same DNA motif. To test this hypothesis, a PEA3 construct containing only the DNA binding domain was cotransfected with pNulit into MDA-MB-453 cells. Expression of the DNA-binding domain of PEA3 (PEA3DBD) resulted in decrease of the luciferase reporter activity in a dose-dependent manner (Fig. 6C). Thus, consistent with the hypothesis, either wild-type PEA3 or the DNA-binding domain of PEA3 can function as potent repressors of the HER-2/neu gene expression .

DISCUSSION

PEA3 as an *ets* family protein

The PEA3 protein contains a domain of about 85 amino acids with extensive sequence similarity with the ETS domain, a conserved region shared by all members of the *ets* family that characteristically bind to the cognate DNA binding site as a monomer through their ETS DNA-binding domain. The consensus DNA binding site of PEA3 seems to occur on the promoters of a cohort of genes and play a positive role for their expression. Examples include the stromelysine gene [37] and the tumor suppressor gene *maspin* [38, 39]. For most of the cases, there are other *ets* proteins rather than the PEA3 protein itself shown to target the consensus binding site and regulate the expression of the genes. The biological effects of the PEA3 protein to these genes remain to be determined. This study, to our knowledge, for the first time shows the tumor suppression function of an *ets* protein through suppressing the oncogenic activity of the HER-2/neu oncogene.

The PEA3 protein downregulates HER-2/neu promoter

We have identified a DNA motif on the HER-2/neu promoter which directly binds to the *ets*-related transcription factor PEA3 resulting in downregulation of the HER-2/neu gene and consequently inhibition of cell transformation *in vitro* and tumor development *in vivo*. Our results indicated that PEA3 can act as a transcriptional repressor and likely compete with other transactivators through the same DNA motif. Since some other *ets* family members which share the same binding site specificity of PEA3 may also bind to the same PEA3 binding site on the HER-2/neu promoter and transactivate the gene, it is conceivable that PEA3 may repress the HER-2/neu promoter by blocking the binding of other transactivating *ets* proteins. There are precedents of other *ets* family members, like ERF and Net, acting as repressors of gene expression [40, 41]. On the other hand, promoters

negatively regulated by Ets binding sites have also been reported [42, 43].

The effect of PEA3 on HER-2/neu promoter in this study is observed mainly in human breast and ovarian cancer cell lines, which are biologically relevant to the diseases. Other groups have previously reported transactivation of HER-2/neu promoter by PEA3 in COS-1 cells [44]. Under our experimental condition, in COS-1 cells we did notice a very weak PEA3-mediated induction of HER-2/neu promoter. The potential species- or cell type-specific transactivating effect of PEA3 is an interesting phenomenon. It might be due to the disparity of regulatory mechanisms between different cell types and/or species. In this regard, it is worth noting that COS cells were derived from monkey kidney cells transformed by SV40 T antigen [45].

The PEA3 gene as a potential gene therapy agent

Recently targeting the protein product of HER-2/neu p185 with a humanized anti-p185 monoclonal antibody (Herceptin) has shown encouraging therapeutic effects for patients with HER-2/neu-overexpressing breast cancer though with significant side effect of cardiotoxicity [46, 47]. The results, however, indicate that HER-2/neu overexpression is an excellent target to develop therapeutic strategies of cancer. In addition to the immunotherapy strategy, a gene therapy setting using either the adenovirus type 5 E1A or the SV40 large T antigen gene to demonstrate HER-2/neu overexpression has been reported [12, 26, 27, 29, 30, 48]. In the case of E1A, a phase I clinical trial has just been completed with observation of HER-2/neu downregulation associated with E1A expression [49]. However, both E1A and SV40 large T antigen are viral proteins and very likely suppress HER-2/neu in an indirect manner as none of these proteins have been shown to bind the HER-2/neu promoter directly. The fact that PEA3 directly binds to the HER-2/neu promoter likely provides stronger efficacy and higher specificity to inhibit HER-2/neu promoter activity. We have presented the data to demonstrate that the DNA-binding domain of PEA3 is sufficient to downregulate HER-2/neu overexpression. These

features make PEA3 an attractive candidate for further molecular manipulation to develop next generation therapeutic molecules with higher binding affinity and enhanced specificity. In addition, the strategy described in this study provides a general approach to identify potential transformation suppressors through searching for DNA-binding protein recognizing specific DNA sequence on the promoter of the targeted oncogenes or other disease-causing pathogenic genes.

METHODS

Plasmids To construct the pNeulit plasmid , the promoter region of human HER-2/neu was amplified with a pair of primers (primer A 5' - GATAGGATCCGGGGGTCCTGGAAGCC-3' and primer B 5' - GGGCAGATCTGGTTTTCCGGTCCCAATGGA-3'). The amplified DNA fragment was digested with BamHI and BglII, and ligated into the BamHI site of pGL2-Basic (Promega, Madison, WI). The sequence of this insert has been confirmed.
For site-directed mutagenesis of the PEA3 binding site, two more primers with sequences spanning the PEA3 binding site on the HER-2/neu promoter were synthesized in which the PEA3 motif AGGAAG was changed to AGCTCG (primer C 5'- GGAGGAGGAGGGCTGCTTGAGCTCGTATAAGAATG-3' and primer D 5'-CATTCTTATACGAGCTCAAGCTCCTCC-3'). The PCR product amplified by primers A and D and the product amplified by primers B and C were annealed followed by Klenow extension. The double-stranded DNA fragment was then cloned into the pGL2-Basic vector as the wild-type promoter. The full-length PEA3 cDNA was cloned into pcDNA3 between the HindIII and BamHI sites (pcDNA3-PEA3). The pSRαPEA3 plasmid used in the focus forming assay contains the same PEA3 cDNA cloned into the pcDL-SRα296 vector and is under the control of a promoter containing the simian virus 40 early promoter and the R-U5 region of the long terminal repeat of human T-cell leukemia virus type 1 [50]. The plasmid carrying the DNA-binding domain of PEA3 (PEA3DBD) was PCR-amplified and cloned into pcDNA3.1/His vector.

Cells Mouse fibroblast NIH3T3 and human cancer cells used in this study were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % bovine calf serum and 10 % fetal bovine serum, respectively.

Gel mobility shift assay The annealed double-strand oligonucleotide derived from the HER-2/neu promoter containing either the wild-type (5'- GGAGCTCGAGGGCTGCTTGAGGAAGTATAAGAATG-3' and the complementary strand) or a mutated PEA3 binding site (5'- GGAGCTCGAGGGCTGCTTGAGCTCGTATAAGAATG-3' and the complementary strand) was end labeled by ^{32}P-γATP. Binding of the protein factors to DNA sequences was achieved in a mixture containing 1X binding buffer (20 mM HEPES, pH 7.9, 5 mM

$MgCl_2$, 5 % glycerol, 0.1 M KCl, 0.2 mM EDTA, 2 mM DTT), 0.3 μg GST-PEA3 (or GST alone or 1 μg nuclear extract of MDA-MB-453), 2 to 8 μg poly d(I-C), 1 μg of bovine serum albumin, and 20,000 cpm of ^{32}P-labeled oligonucleotide. 100× the amount of competitor oligonucliotide was added when necessary. In the protein competition assay, 0.3 or 0.6 μg of GST-PEA3 protein was included in the reaction mixture. The binding reaction was performed at room temperature for 20 minutes. The samples were separated in a 5 % polyacrymide. Gels were dried under vacuum and autoradiographed.

Transient transfection and luciferase assay Human cancer cells was seeded with 60-80 % of confluency and transfected by incubating with DNA : DC-chol liposome complex (13 nmol lipid : 1 μg DNA) in serum free medium for 2-5 hours, or by incubating with the DNA : polyethylenimine (PEI, 4.5 μg/μl, average MW 25,000; Aldrich, Milwaukee, WI) complex (0.5 μl PEI : 1 μg DNA) for 1-3 hours. Cell lysates were prepared 48 hours later for luciferase activity assay by following the manufacturer's instruction (Promega) using the monolight 2010 Luminometer (Analytical Luminescence Laboratory). The measured luciferase activities were normalized by measuring the co-transfected ß-galactosidase activity. The ß-galactosidase activity was measured by mixing equal amounts of cell extract and 2 X Buffer (120 mM Na_2HPO_4, 80 mM NaH_2PO_4, 2 mM $MgCl_2$, 100 mM ß-mercaptoethanol, 1.33 mg/ml orthonitrophenyl galactose). After incubating at 37°C, enzyme activity was determined by absorbance at 420 nm.

Stable transfection and colony formation assay Human cancer cells under 80 % confluency were transfected with 1 μg of PEA3 expression plasmid or the pcDNA3 vector using DC-chol liposome as a carrier, and subject to neomycin selection. Neomycin-resistant colonies were stained by 1 % crystal violet and counted. Transfections of each cell line were repeated 2-3 times using cells of different passages and plasmid DNA from independent preparations.

Orthotopic ovarian cancer model and liposome-mediated *in vivo* gene transfer Four- to six-week-old athymic female *nu/nu* mice were purchased from Harlan Sprague Dawley, Inc.(Indianapolis, IN) and treated in accordance with

institutional guidelines. To establish tumors, 2×10^6 SKOV-3-ip1 cells were injected into the intraperitoneal (ip) cavity as described [27, 29]. Five days after ip injection of cancer cells, the mice that had tumors were separated in five groups and received weekly ip injections of 200 μl of different reagents for a period of time. The responses and survival rates were observed for 1 year.

Immunoblotting Immunoblot analysis for PEA3 and HER-2/neu p185 expression in cells and tumor samples was performed as described previously [26, 48].

ACKNOWLEDGMENTS

The authors gratefully thank Dr. J H Chen for providing PEA3 cDNA. This work was partially supported by NIH grants RO1-CA58880, RO1-CA77855 (to MCH), RO1-CA64654 (to LH), and supported by M. D. Anderson Breast Cancer Research Program as well as a NIH cancer center core grant (CA 16672). XX is a recipient of predoctoral fellowship from the Breast Cancer Research Program of the Department of Defense (DAMD17-96-1-6223).

FIGURE LEGENDS

Figure 1 PEA3 binds to the HER-2/neu promoter. GST-PEA3 fusion protein was incubated with ^{32}p-γ ATP end-labeled wild-type or mutated oligonucleotide containing the consensus PEA3 binding site sequence on the HER-2/neu promoter (lanes 1). The binding specificity was tested by competition with unlabeled wild-type (lanes 2) or non-specific oligonucleotide (lanes 3).

Figure 2 PEA3 inhibits the HER-2/neu promoter activity in HER-2/neu overexpressing cancer cell lines. Human ovarian cancer cells SKOV-3 were cotransfected with 5 μg of pNeulit or HER2-CAT with different amounts of PEA3 plasmid DNA (pcDNA3-PEA3) as indicated. The luciferase (A) or the chlroamphenicol acetyltransferase (CAT) (B) reproter activity was measured. For the CAT transient assay, the doses of cotransfected pcDNA3-PEA3 were 0, 5, 10, and 15 μg. The results of two independent experiments are shown.

Figure 3 PEA3 represses HER-2/neu promoter activity through a positive regulatory DNA motif. A, Luciferase reporter gene driven by either wild-type (open bars) or the PEA3 site-mutated (close bars) HER-2/neu promoter was measured after co-transfection with PEA3 cDNA into MDA-MB-453 cells. B, The luciferase activities derived from wild-type and the PEA3 site-mutated HER-2/neu promoter were compared by transfection into a breast cancer cell line (MDA-MB-453) and a ovarian cancer cell line (SKOV-3-ip1).

Figure 4 PEA3 represses neu-mediated transformation and inhibits cell growth of

HER-2/neu-overexpressing cancer cells. A, PEA3 represses the transforming activity of activated genomic rat neu. NIH3T3 cells (2×10^5) were plated to a 100-mm culture dish 24 hours before transfection. Cells were transfected with 0.5 μg of pSV2neo, 1 μg of cNu104 plus 5 μg of either pSRαPEA3 or the control vector pSV2E by calcium phosphate precipitation. Two days after transfection, cells were split and two plates were subject to selection in 500 μg/ml of neomycin (G418; Life Technologies, Inc), and the other two were maintained in normal medium until foci appeared. Foci and neomycin-resistant colonies were stained and counted. The number of foci was normalized by the corresponding number of neomycin-resistant colonies. Results are expressed as ratio of the number of foci to the number of colonies from each transfection. The ratio of cNu104 transfection alone was set as 100 %. B, An example of the PEA3-mediated suppression of the focus forming activity by cNu104. C, Cell growth of HER-2/neu-overexpressing breast and ovarian cancer cells was inhibited by PEA3 transfection as indicated by the decrease of colony numbers compared with vector trasfection. D, Ectopic PEA3 RNA expression was preferentially lost in the survival neomycin-resistant clones derived from the HER-2/neu-overexpressing breast cancer cell line MDA-MB-453. Total RNA were extracted from cell clones resulted from stable transfection and RT-PCR was performed by following the manufacturer's instruction (SuperScript preamplification system; Life Technologies, Inc). The primers derived from the PEA3 coding sequence (5'-TGAATTATGACAAGCTGAGCCG-3') and from the expression vector pcDNA3 (5'-TCAGCGAGCTCTAGCATTTAGG-3') were use to amplified the ectopically expressed PEA3 transcript. Primers for GAPDH internal control were: 5'-AGGTGAAGGTCGGAGTCAAC-3' and 5'-TCCATTGATGACAAGCTTCCC-3'. COS-1 cells transiently transfected by the PEA3 cDNA were used as a positive control. Amplification was performed on a Perkin Elmer DNA Cycler 480 for 35 cycles with

denaturing at 94°C for 30 seconds, annealing at 58°C for 1.5 minutes and extension at 72°C for 1.5 minutes.

Figure 5 PEA3 inhibits growth of tumor xenografts derived from HER-2/neu-overexpressing cancer cells. A, Nude mice carrying intraperitoneal tumors derived from SKOV3-ip1 received weekly ip injections of a reagent containing PEA3 plasmid DNA complexed with liposome (PEA3-liposome), control DNA (pGEM) complexed with liposome (pGEM-Liposome), naked PEA3 plasmid DNA, or PBS. Number of mice in each group is shown in the parentheses. The arrow marks the last injection. Tumor samples were retrieved from mice treated with PBS, the lipid vehicle DC-Chol, PEA3 naked DNA, or PEA3-DC-Chol complex, and the levels of p185 were measured by immunoblotting as shown in the insert. Loading was controlled by the endogenous α-actin expression (data not shown). B, Nude mice carrying tumors derived from 2774 10-c were treated with PEA3-liposome complex (the solid line) or PBS alone (the dashed line) by the same procedure as SKOV-3-ip1 cells.

Figure 6 PEA3 competes with a distinct family member of the *ets* family detected in HER-2/neu-overexpressing cancer cells. A, Nuclear extract of MDA-MB-453 cells was incubated with the DNA oligonucleotide probe in the absence (Oligo) or presence of unlabeled competitor of the same specific oligonucleotide (SC) or non-specific oligonucletide of shuffled sequences (NSC). The signal of specific DNA-protein complex is indicated by an arrow. B, Increasing amount of GST-PEA3 fusion protein was added into the binding reaction to test the competition between the unknown nuclear factor and PEA3 protein. The DNA-PEA3 complex is indicated by an arrow. NE, nuclear extract. C, The DNA-binding domain of PEA3 is sufficient to down-regulate the HER-2/neu promoter as assayed by cotransfection of different doses of PEA3DBD and

pNulite. The result from a representative experiment is shown.

Table I. PEA3 suppresses growth of cancer cells overexpressing HER-2/neu.[1]

HER-2/neu overexpressers

Cell lines	pcDNA3	PEA3
SK-BR-3	100	0.7 (±0.9)
MDA-MB-453	100	19.7 (±3.0)
SKOV3-ip1	100	14.3 (±4.1)

HER-2/neu basal-level expressers

Cell lines	pcDNA3	PEA3
MDA-MB-435	100	73.4 (±10.9)
2774 c-10	100	83.3 (±3.8)

[1] Experiment for each cell line was independently repeated 2-4 times using different batches of plasmid DNA and cell culture. Inhibition of colony formation by PEA3 transfection is shown in percentage with the number of neomycin-resistant colony resulted from pcDNA3 transfection arbitrarily set as 100 %.

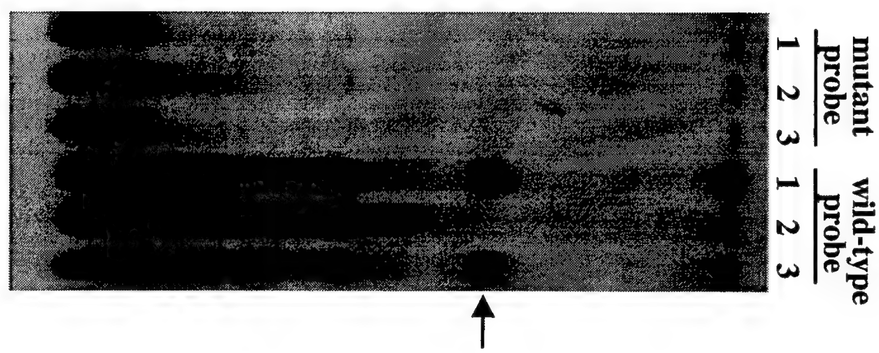
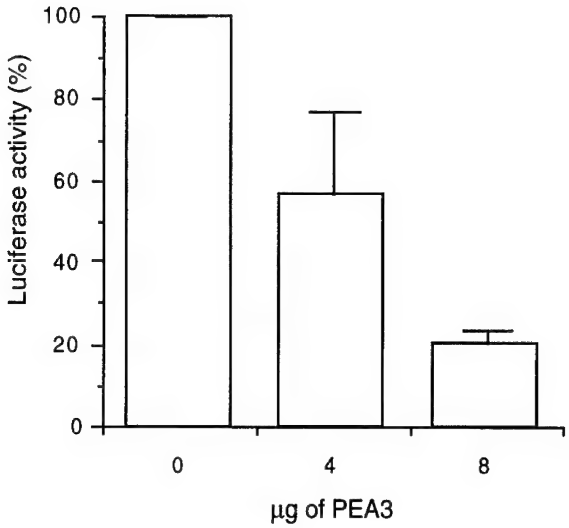


Fig. 1

A.


100
80
60
40
20
0
Luciferase activity (%)
0
4
8
µg of PEA3

B.

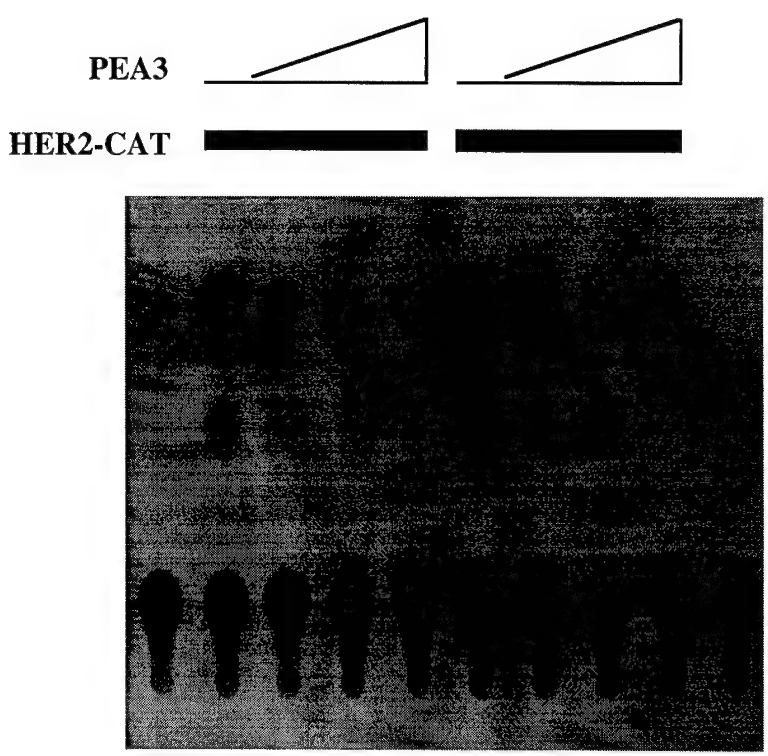

PEA3
HER2-CAT

Fig. 2

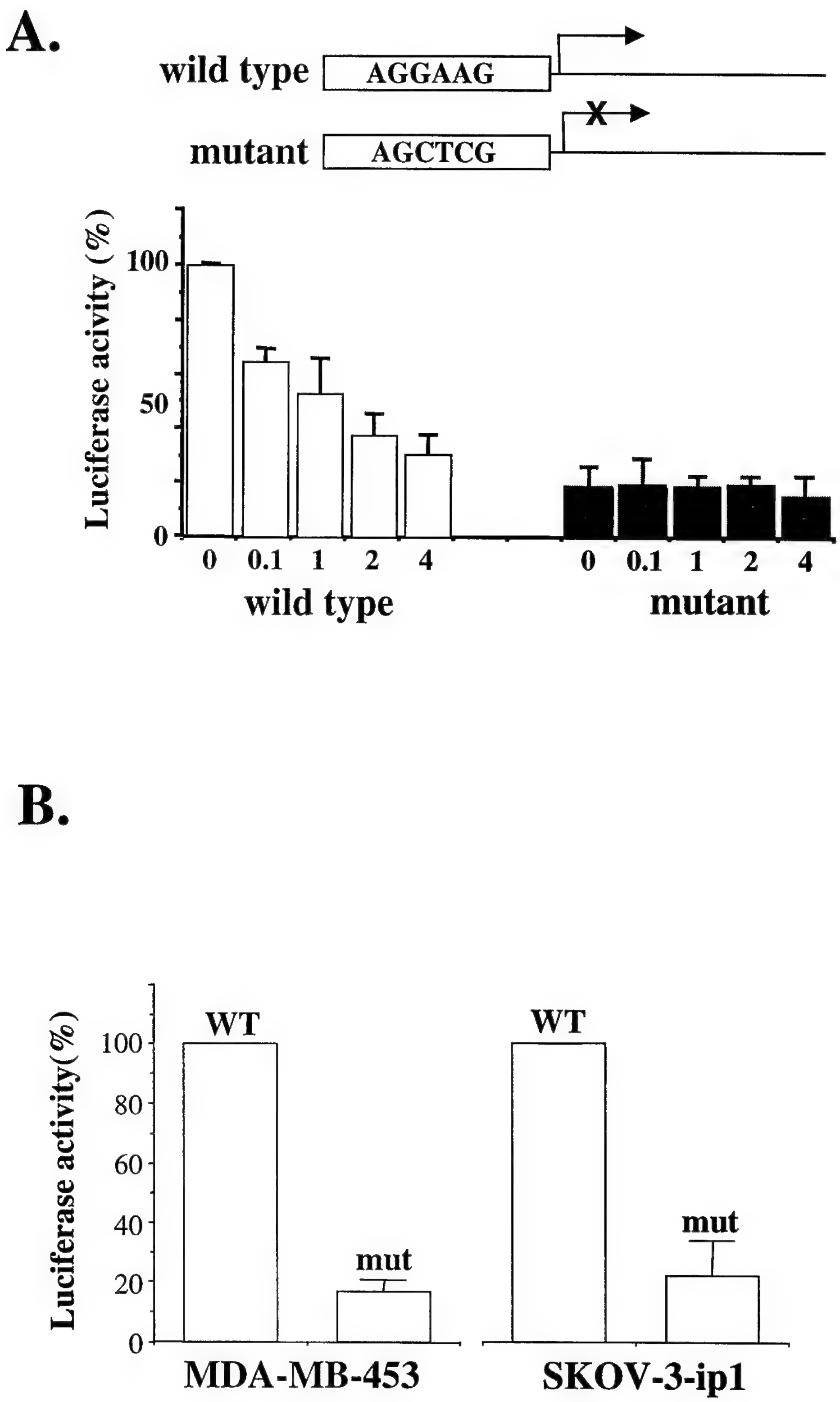

A.
wild type
AGGAAG
mutant
AGCTCG
Luciferase acivity (%)
100
50
0
0
0.1
1
2
4
wild type
0
0.1
1
2
4
mutant
B.
Luciferase activity(%)
100
80
60
40
20
0
WT
mut
WT
mut
MDA-MB-453
SKOV-3-ip1

Fig. 3

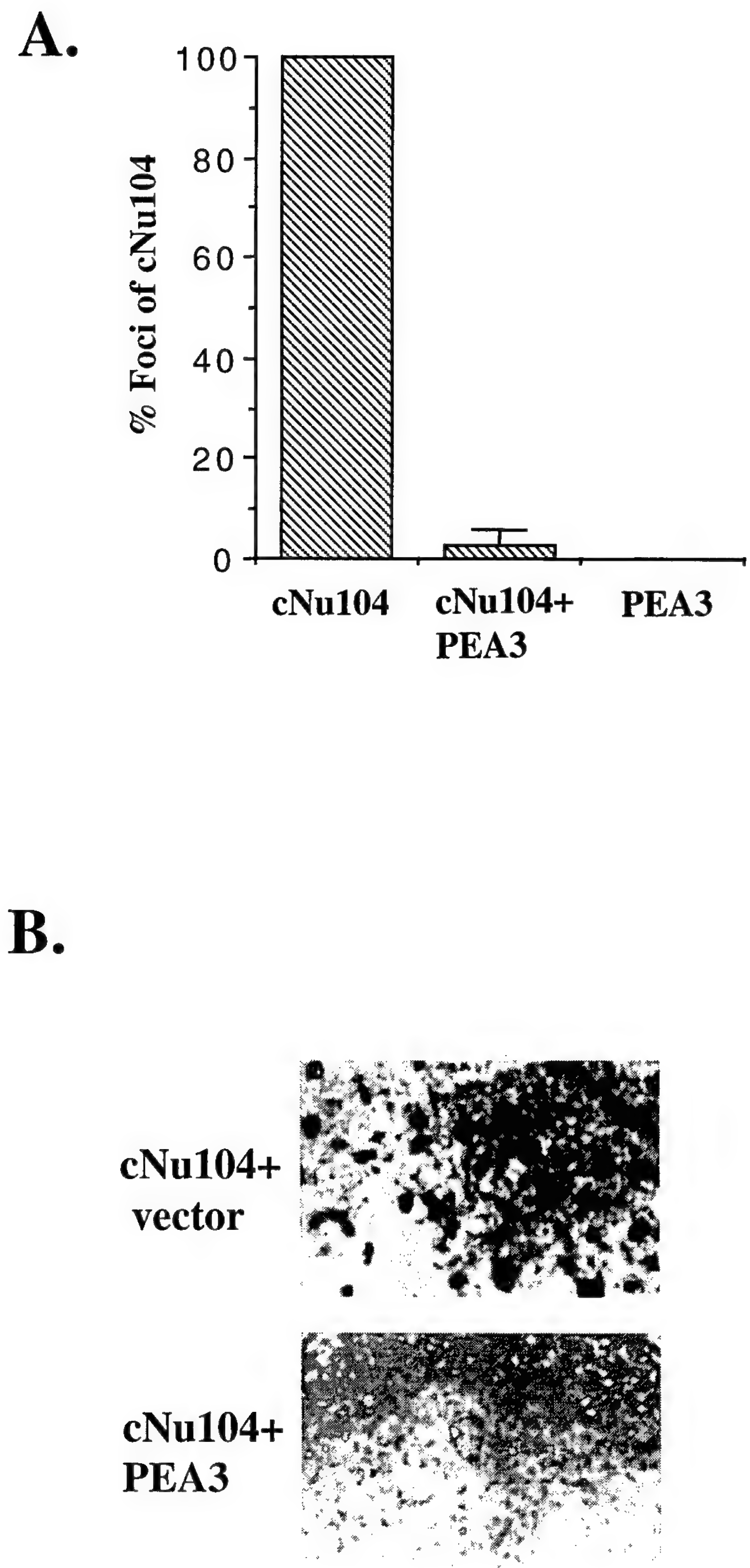

A.
100
80
60
40
20
0
% Foci of cNu104
cNu104
cNu104+
PEA3
PEA3
B.
cNu104+
vector
cNu104+
PEA3

Fig. 4

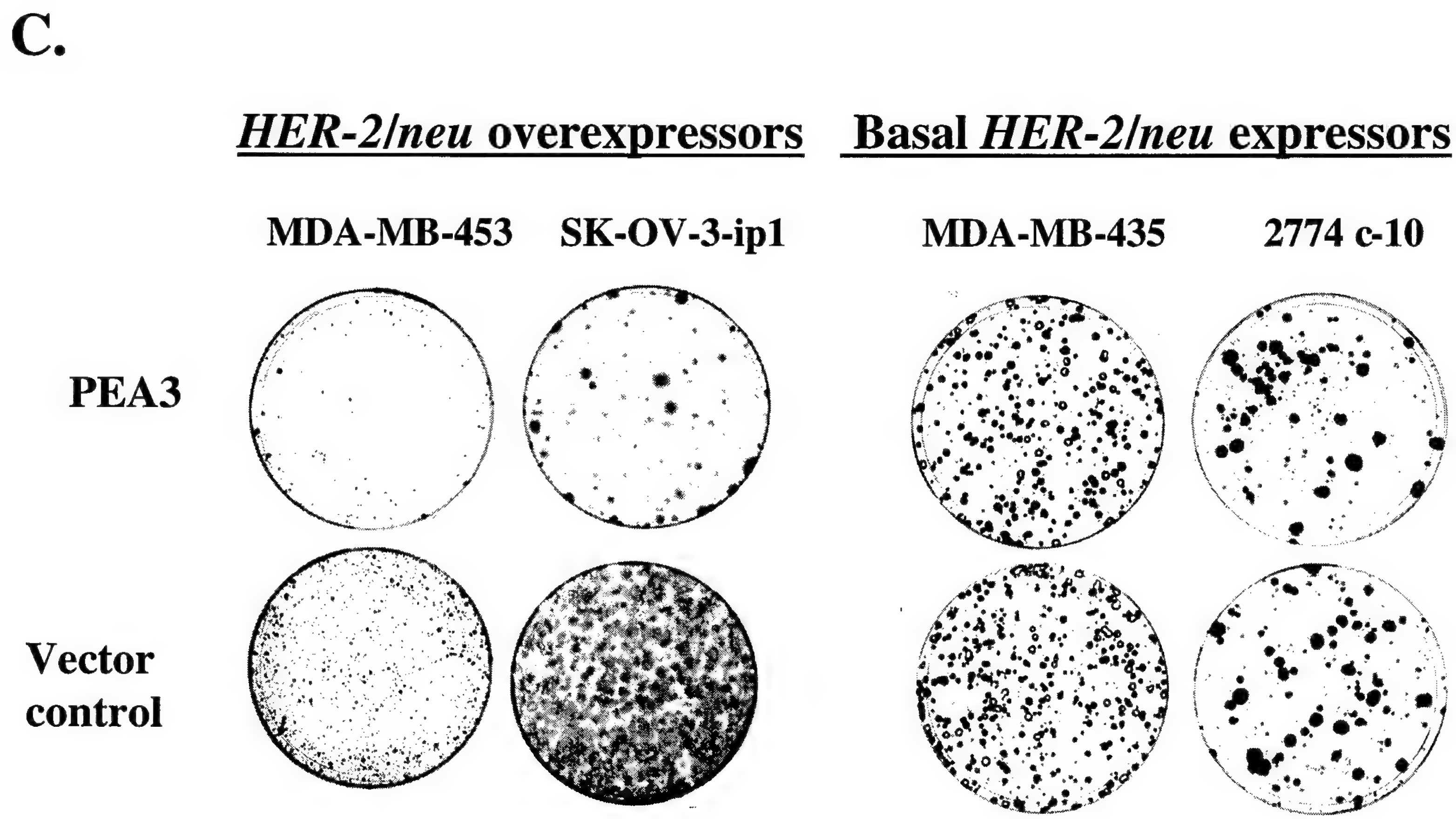

C.
HER-2/neu overexpressors
Basal HER-2/neu expressors
MDA-MB-453
SK-OV-3-ip1
MDA-MB-435
2774 c-10
PEA3
Vector control

Fig. 4

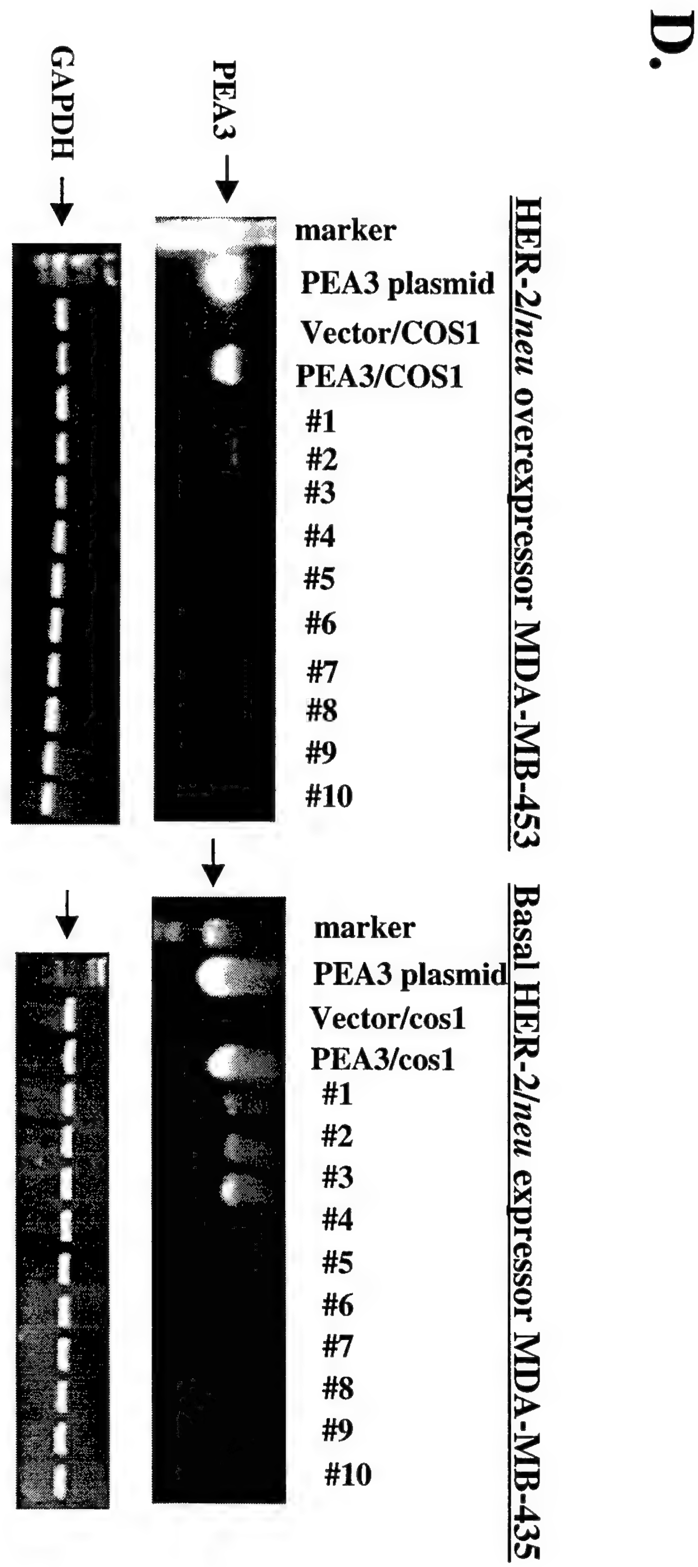

D.
PEA3
GAPDH
HER-2/neu overexpressor MDA-MB-453
marker
PEA3 plasmid
Vector/COS1
PEA3/COS1
#1
#2
#3
#4
#5
#6
#7
#8
#9
#10
Basal HER-2/neu expressor MDA-MB-435
marker
PEA3 plasmid
Vector/cos1
PEA3/cos1
#1
#2
#3
#4
#5
#6
#7
#8
#9
#10

Fig. 4

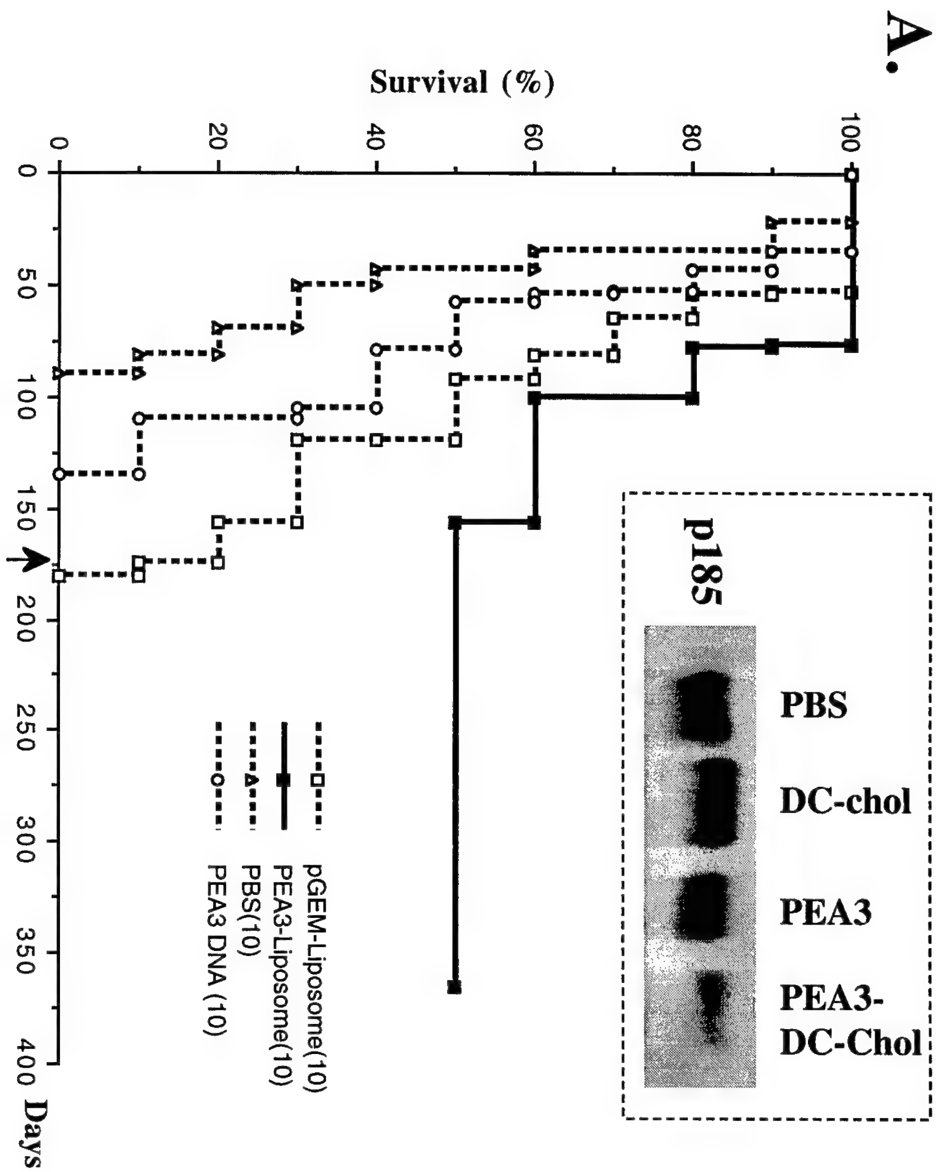
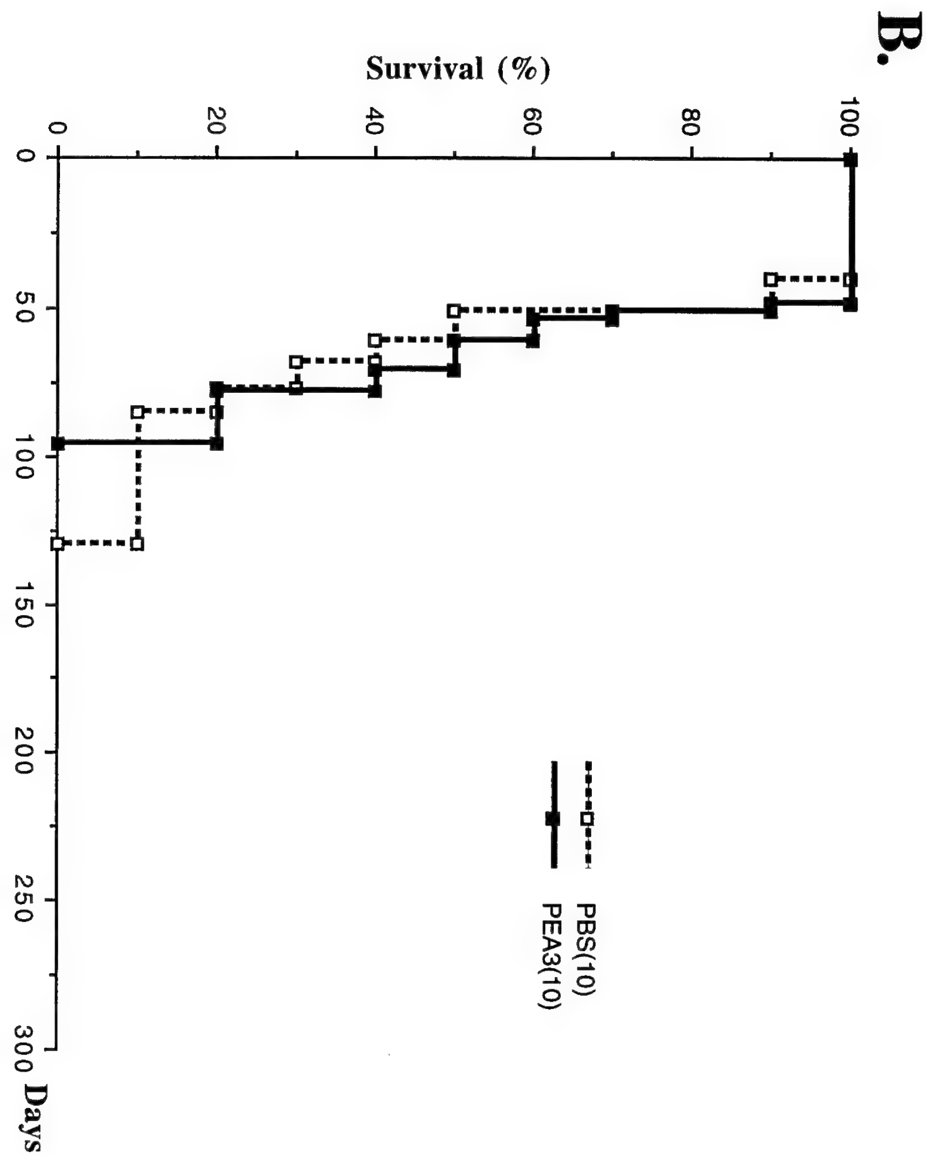

A.
Survival (%)
Days
p185
PBS
DC-chol
PEA3
PEA3-
DC-Chol
pGEM-Liposome(10)
PEA3-Liposome(10)
PBS(10)
PEA3 DNA (10)

Fig. 5



B.
Survival (%)
0
20
40
60
80
100
0
50
100
150
200
250
300
Days
PBS(10)
PEA3(10)

Fig.5

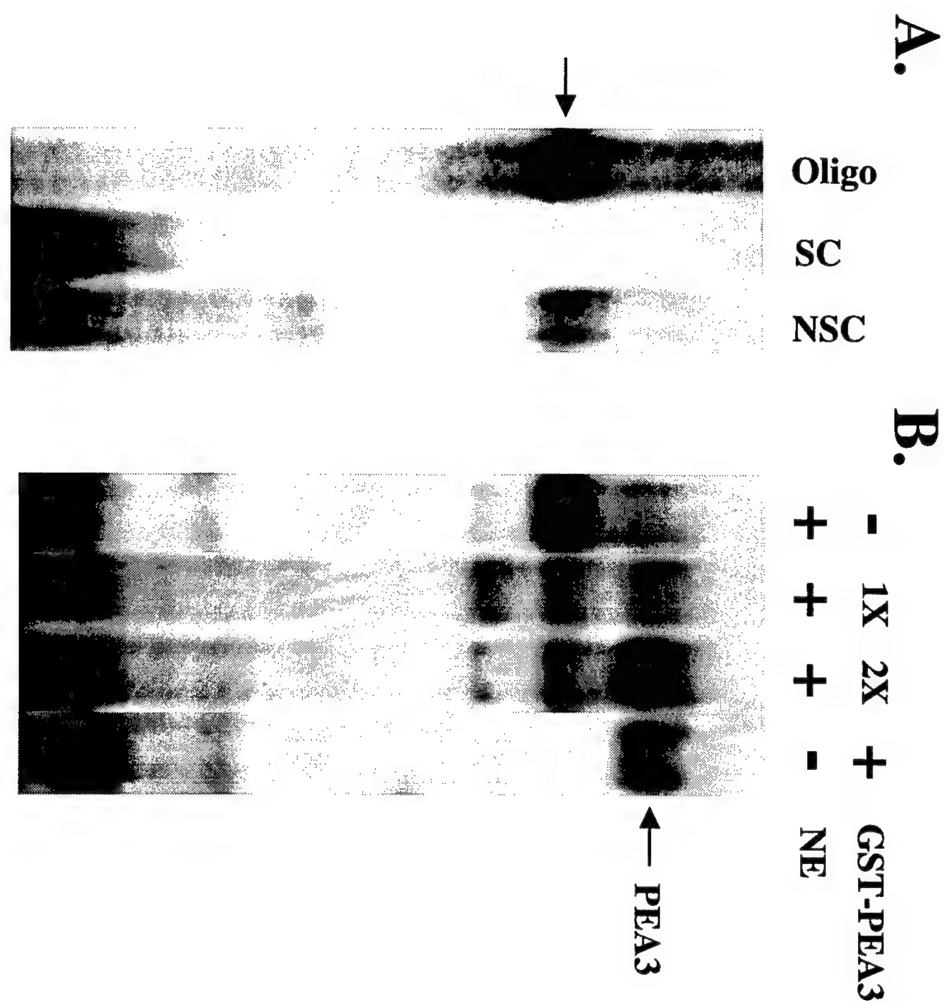


Fig. 6

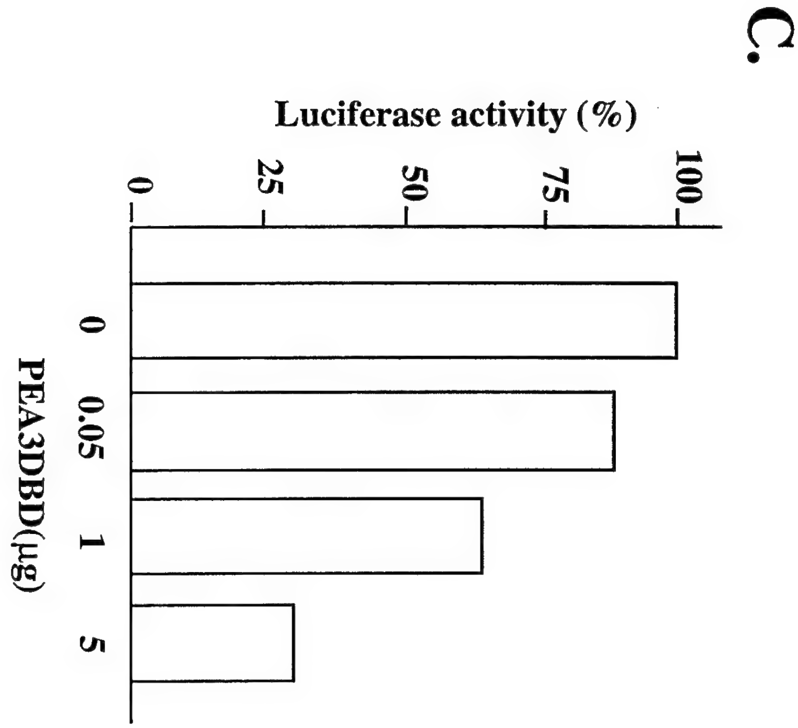


Fig. 6

REFERENCES

1. Park, J.B., Rhim, J.S., Park, S.C., Kimm, S.W. & Kraus, M.H. Amplification, overexpression, and rearrangement of the erbB-2 protooncogene in primary human stomach carcinomas. *Cancer Research* **49**, 6605-6609 (1989).
2. Schneider, P.M. *et al.* Differential expression of the c-erbB-2 gene in human small cell and non-small cell lung cancer. *Cancer Research* **49**, 4968-4971 (1989).
3. Weiner, D.B. *et al.* Expression of the neu gene-encoded protein (P185neu) in human non-small cell carcinomas of the lung. *Cancer Research* **50**, 421-425 (1990).
4. Xia, W., Lau, Y.K., Zhang, H.Z. & Hung, M.-C. Strong correlation between c-erbB-2 overexpression and overall survival of patients with oral squamous cell carcinoma. *Clinical Cancer Research* **3**, 3-9 (1997).
5. Yokota, J. *et al.* Genetic alterations of the c-erbB-2 oncogene occur frequently in tubular adenocarcinoma of the stomach and are often accompanied by amplification of the v-erbA homologue. *Oncogene* **2**, 283-287 (1988).
6. Shi, D. *et al.* Overexpression of the c-erbB-2/neu-encoded p185 protein in primary lung cancer. *Molecular Carcinogenesis* **5**, 213-218 (1992).
7. D'Emilia, J. *et al.* Expression of the c-erbB-2 gene product (p185) at different stages of neoplastic progression in the colon. *Oncogene* **4**, 1233-1239 (1989).
8. Slamon, D.J. *et al.* Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* **235**, 177-182 (1987).
9. Slamon, D.J. *et al.* Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* **244**, 707-712 (1989).
10. Zhang, X., Silva, E., Gershenson, D. & Hung, M.C. Amplification and

rearrangement of c-erb B proto-oncogenes in cancer of human female genital tract. *Oncogene* **4**, 985-989 (1989).

11. Berchuck, A. *et al.* Overexpression of HER-2/neu is associated with poor survival in advanced epithelial ovarian cancer. *Cancer Research* **50**, 4087-4091 (1990).

12. Yu, D. & Hung, M.C. The erbB2 gene as a cancer therapeutic target and the tumor- and metastasis-suppressing function of E1A. *Cancer & Metastasis Reviews* **17**, 195-202 (1998).

13. Chazin, V.R., Kaleko, M., Miller, A.D. & Slamon, D.J. Transformation mediated by the human HER-2 gene independent of the epidermal growth factor receptor. *Oncogene* **7**, 1859-1866 (1992).

14. Yu, D.H. & Hung, M.-C. Expression of activated rat neu oncogene is sufficient to induce expreimental metastasis in 3T3 cells. *Oncogene* **6**, 1991-1996 (1991).

15. Yu, D. *et al.* c-erbB-2/neu overexpression enhances metastatic potential of human lung cancer cells by induction of metastasis-associated properties. *Cancer Research* **54**, 3260-3266 (1994).

16. Benz, C.C. *et al.* Estrogen-dependent, tamoxifen-resistant tumorigenic growth of MCF-7 cells transfected with HER2/neu. *Breast Cancer Research & Treatment* **24**, 85-95 (1993).

17. Tan, M., Yao, J. & Yu, D. Overexpression of the c-erbB-2 gene enhanced intrinsic metastasis potential in human breast cancer cells without increasing their transformation abilities. *Cancer Research* **57**, 1199-1205 (1997).

18. Tsai, C.M. *et al.* Correlation of intrinsic chemoresistance of non-small-cell lung cancer cell lines with HER-2/neu gene expression but not with ras gene mutations. *Journal of the National Cancer Institute* **85**, 897-901 (1993).

19. Tsai, C.M. *et al.* Enhanced chemoresistance by elevation of p185neu levels

in HER-2/neu-transfected human lung cancer cells. *Journal of the National Cancer Institute* **87**, 682-684 (1995).

20. Yu, D. *et al.* Overexpression of c-erbB-2/neu in breast cancer cells confers increased resistance to Taxol via mdr-1-independent mechanisms. *Oncogene* **13**, 1359-1365 (1996).

21. Yu, D. *et al.* Overexpression of ErbB2 blocks Taxol-induced apoptosis by upregulation of p21Cip1, which inhibits p34Cdc2 kinase. *Molecular Cell* **2**, 581-591 (1998).

22. Yu, D. *et al.* Overexpression of both p185c-erbB2 and p170mdr-1 renders breast cancer cells highly resistant to taxol. *Oncogene* **16**, 2087-2094 (1998).

23. Yu, D., Suen, T.C., Yan, D.H., Chang, L.S. & Hung, M.C. Transcriptional repression of the neu protooncogene by the adenovirus 5 E1A gene products. *Proceedings of the National Academy of Sciences of the United States of America* **87**, 4499-4503 (1990).

24. Yu, D., Hamada, J., Zhang, H., Nicolson, G.L. & Hung, M.C. Mechanisms of c-erbB2/neu oncogene-induced metastasis and repression of metastatic properties by adenovirus 5 E1A gene products. *Oncogene* **7**, 2263-2270 (1992).

25. Yu, D., Suen, T.C., Yan, D.H., Chang, L.S. & Hung, M.C. Transcriptional repression of the neu protooncogene by the adenovirus 5 E1A gene products. *Oncogene* **7**, 2263-2270 (1992).

26. Zhang, Y., Yu, D., Xia, W. & Hung, M.C. HER-2/neu-targeting cancer therapy via adenovirus-mediated E1A delivery in an animal model. *Oncogene* **10**, 1947-1954 (1995).

27. Xing, X. *et al.* Mutant SV40 large T antigen as a therapeutic agent for HER-2/neu-overexpressing ovarian cancer. *Cancer Gene Therapy* **3**, 168-174 (1996).

28. Chen, H. & Hung, M.-C. Involvement of co-activator p300 in the transcriptional regulation of the HER-2/neu gene. *J. Biol. Chem.* **272**, 6101-6104

(1997).

29. Yu, D. *et al.* Liposome-mediated in vivo E1A gene transfer suppressed dissemination of ovarian cancer cells that overexpress HER-2/neu. *Oncogene* **11**, 1383-1388 (1995).

30. Hung, M.C. *et al.* HER-2/neu-targeting gene therapy--a review. *Gene* **159**, 65-71 (1995).

31. Xin, J.H., Cowie, A., Lachance, P. & Hassell, J.A. Molecular cloning and characterization of PEA3, a new member of the Ets oncogene family that is differentially expressed in mouse embryo cells. *Genes Dev.* **6**, 481-496 (1992).

32. Tal, M. *et al.* Human HER2 (neu) promoter: evidence for multiple mechanisms for transcriptional initiation. *Mol. Cell Biol.* **7**, 2597-2601 (1987).

33. White, M.R. & Hung, M.C. Cloning and characterization of the mouse neu promoter. *Oncogene* **7**, 677-683 (1992).

34. Hung, M.-C., Schechter, A.L., Chevray, P.L., Stern, D.F. & Weinberg, R.A. Molecular cloning of the neu gene: absence of gross structural alteration in oncogenic alleles. *Proc. Natl. Acad. Sci. USA* **83**, 261-264 (1986).

35. Bargmann, C.I., Hung, M.C. & Weinberg, R.A. Multiple independent activations of the neu oncogene by a point mutation altering the transmembrane domain of p185. *Cell* **45**, 649-657 (1986).

36. Yu, D.H., Scorsone, K. & Hung, M.C. Adenovirus type 5 E1A gene products act as transformation suppressors of the neu oncogene. *Molecular & Cellular Biology* **11**, 1745-1750 (1991).

37. Buttice, G. & Kurkinen, M. A polyomavirus enhancer A-binding protein-3 site and Ets-2 protein have a major role in the 12-O-tetradecanoylphorbol-13-acetate response of the human stromelysine gene. *J. Biol. Chem.* **268**, 7196-7204 (1993).

38. Zhang, M., Magit, D. & Sager, R. Expression of maspin in prostate cells is

regulated by a positive ets element and a negative hormonal responsive element site recognized by androgen receptor. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 5673-5678 (1997).

39. Zhang, M., Maass, N., Magit, D. & Sager, R. Transactivation through Ets and Ap1 transcription sites determines the expression of the tumor-suppressing gene maspin. *Cell Growth & Differentiation* **8**, 179-186 (1997).

40. Sgouras, D.N. *et al.* ERF: an ETS domain protein with strong transcriptional repressor activity, can suppress ets-associated tumorigenesis and is regulated by phosphorylation during cell cycle and mitogenic stimulation. *EMBO Journal* **14**, 4781-4793 (1995).

41. Giovane, A., Pintzas, A., Maira, S.M., Sobieszczuk, P. & Wasylyk, B. Net, a new ets transcription factor that is activated by Ras. *Genes & Development* **8**, 1502-1513 (1994).

42. Chen, H.M. & Boxer, L.M. Pi 1 binding sites are negative regulators of bcl-2 expression in pre-B cells. *Molecular & Cellular Biology* **15**, 3840-3847 (1995).

43. Goldberg, Y., Treier, M., Ghysdael, J. & Bohmann, D. Repression of AP-1-stimulated transcription by c-Ets-1. *Journal of Biological Chemistry* **269**, 16566-16573 (1994).

44. Benz, C.C. *et al.* HER2/Neu and the Ets transcription activator PEA3 are coordinately upregulated in human breast cancer. *Oncogene* **15**, 1513-1525 (1997).

45. Gluzman, Y. SV40-transformed simian cells support the replication of early SV40 mutants. *Cell* **23**, 175-182 (1981).

46. Baselga, J., Norton, L., Albanell, J., Kim, Y.M. & Mendelsohn, J. Recombinant humanized anti-HER2 antibody (Herceptin) enhances the antitumor activity of paclitaxel and doxorubicin against HER2/neu overexpressing human breast cancer xenografts. *Cancer Research* **58**, 2825-2831 (1998).

47. Pegram, M.D. *et al.* Phase II study of receptor-enhanced chemosensitivity using recombinant humanized anti-p185HER2/neu monoclonal antibody plus cisplatin in patients with HER2/neu-overexpressing metastatic breast cancer refractory to chemotherapy treatment. *Journal of Clinical Oncology* **16**, 2659-2671 (1998).

48. Chang, J.Y. *et al.* The tumor suppression activity of E1A in HER-2/neu-overexpressing breast cancer. *Oncogene* **14**, 561-568 (1997).

49. Hung, M.-C., Wang, S.-C. & Hortobagyi, G. Targeting HER-2/neu-overexpressing cancer cells with transcriptional repressor genes delivered by cationic liposome. in *Non-viral vectors for gene therapy* (eds Huang, L., Hung, M.-C. & Wagner, E.) (Academic Press, San Diego, CA, 1999, in press).

50. Takebe, Y. *et al.* SRa promoter: an efficient and versatile mammalian cDNA expression system composed of the simian virus 40 early promoter and the R-U5 segment of human T-cell leukemia virus type 1 long terminal repeat. *Mol. Cell. Biol.* **8**, 466-472 (1988).